UNITED STATES PATENT AND TRADEMARK OFFICE

UNITED STATES DEPARTMENT OF COMMERCE
United States Patent and Trademark Office
Address: COMMISSIONER FOR PATENTS
P.O. Box 1450
Alexandria, Virginia 22313-1450
www.uspto.gov

APPLICATION NO.	FILING DATE	FIRST NAMED INVENTOR	ATTORNEY DOCKET NO.	CONFIRMATION NO.
09/484,964	01/18/2000	Edward T. H. Yeh	UTSH.248	1333

7590 07/16/2003

FULBRIGHT & JAWORSKI, LLP
600 CONGRESS AVE
SUITE 2400
AUSTIN, TX 78701

EXAMINER
WEHBE, ANNE MARIE SABRINA

ART UNIT	PAPER NUMBER
1632	20

DATE MAILED: 07/16/2003

Please find below and/or attached an Office communication concerning this application or proceeding.

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office
ASSISTANT SECRETARY AND COMMISSIONER OF
PATENTS AND TRADEMARKS
Washington, D.C. 20231

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Paper No. 20

Application Number: 09/484,964
Filing Date: January 18, 2000
Appellant(s): Edward T.H. Yeh

MAILED
JUL 16 2003
GROUP 2900

Gina N. Shishima

For Appellant

EXAMINER'S ANSWER

This is in response to appellant's supplemental brief on appeal filed 5/2/03.

(1) Real Party in Interest

A statement identifying the real party in interest is contained in the brief.

(2) Related Appeals and Interferences

A statement identifying the related appeals and interferences which will directly affect or be directly affected by or have a bearing on the decision in the pending appeal is contained in the brief.

(3) Status of Claims

The statement of the status of the claims contained in the brief is correct.

(4) Status of Amendments After Final

The appellant's statement of the status of amendments after final rejection contained in the brief is correct.

(5) Summary of Invention

The summary of invention contained in the brief is correct.

(6) Issues

The appellant's statement of the issues in the brief is substantially correct in that certain claims have been rejected under 35 U.S.C. 112, first paragraph, for lack of written description, and that certain claims have been rejected under 35 U.S.C. 112, first paragraph for lacking sufficient enablement. However, only claims 73-75, 86-92, 94, and 96-101 are rejected for lack of written description. Claim 85 was never rejected for lack of written description. Further, the rejection of claim 95 for lack of written description has been withdrawn. Therefore, the issue of whether the specification contains sufficient written description of the instant invention only applies to claims 73-75, 86-92, 94, and 96-101. Regarding the issue of enablement, the rejection of record is a **scope of enablement** rejection over all pending claims 73-75, 85-92, and 94-101.

(7) Grouping of Claims

Appellant's brief includes a statement that claim 85 does not stand or fall together with the remaining claims in regards to the enablement rejection, and that claim 95 does not stand or fall together with the remaining claims in regards to the written description rejection. However, the appellant's statement in the brief that certain claims do not stand or fall together is not agreed. As discussed in detail in section (6) above, the written description rejection has never been applied to claim 85 and has been withdrawn over claim 95. Thus, claims 85 and 95 are only rejected under 35 U.S.C. 112, first paragraph, for lack of enablement for the scope of invention as claimed. Furthermore, in regards to claim 85, the appellant misstates the grounds of rejection for lack of enablement. The method recited in claim 85 has been rejected for lack of enablement based several issues, including 1) the lack of enablement in the specification for inhibiting any pathway of apoptosis other than Fas or TNFRI mediated apoptosis *in vitro* or *in vivo*, and 2) the lack of enablement for inhibiting any pathway of apoptosis *in vivo*. These grounds of rejection are shared by claims 73-75, 86-92, and 94-101. Therefore, claim 85 is **not** separately patentable from claims 73-75, 86-92, 94-101 in regards to the enablement rejection.

(8) Claims Appealed

The copy of the appealed claims contained in the Appendix to the brief is correct.

(9) Prior Art of Record

The following is a listing of the prior art and post filing art of record relied upon in the rejection of claims under appeal.

Lavin et al. "Role of protein kinase activity in apoptosis ". Experientia, Vol. 52 (1996), pp. 979-994.

Lieberthal et al. "Mechanisms of apoptosis and its potential role in renal tubular epithelial cell injury". Am. J. Phys., Vol. 271 (3 part 2) (1996), pp. F477-F488.

Marshall "Gene Therapy's Growing Pains". Science, Vol. 269 (1995), pp. 1050-1055.

Orkin et al. "Report and recommendations of the panel to assess the NIH investment in research on gene therapy". (1995). pp. 1-25.

Verma, I. M., et al. "Gene therapy-promises, problems, and prospects". Nature, Vol. 389 (September 1997), pp. 239-242.

10) Grounds of Rejection

The following ground(s) of rejection are applicable to the appealed claims:

A. Claims 73-75, 86-92, 94, and 96-101 stand rejected under 35 U.S.C. 112, first paragraph, for lack of written description. This rejection is set forth below and in prior Office actions mailed on 9/28/01 and 6/5/02, Paper Nos. 10 and 13 respectively. Please note that the rejection of record has been withdrawn over claim 95 in view of applicant's argument that claim 95 contains the limitation that the nucleic acid segment comprises at least SEQ ID NO:1.

The previous office actions set forth the following rejection. The specification does not provide a sufficient written description for a human sentrin-1 gene or polypeptide which has a nucleotide sequence other than SEQ ID NO:1 or an amino acid sequence other than SEQ ID NO:2. The rejected claims contain two independent claims. The broadest independent claim is claim 92 which recites a method of inhibiting apoptosis in a cell comprising providing the cell with a nucleic acid segment comprising at least about 100 contiguous nucleotides of SEQ ID NO:1. At its broadest interpretation, this claim reads on nucleic acids which share no similarity whatsoever with SEQ ID NO:1 except for 100 contiguous nucleotides. Claims 94, and 96-101 depend on

claim 92. Independent claim 73 recites a method of inhibiting apoptosis in a cell comprising providing the cell with a nucleic acid segment encoding a human sentrin-1 polypeptide, wherein the segment encodes at least 100 contiguous amino acids of SEQ ID NO:2. Claims 74-75, and 86-91 depend on claim 73. The specification discloses a single nucleic acid sequence identified as SEQ ID NO:1, which comprises an open reading frame which encodes an amino acid sequence identified as SEQ ID NO:2. The specification further identifies SEQ ID NO:2 as human sentrin-1 and SEQ ID NO:1 as the nucleic acid sequence encoding human sentrin-1. While the specification discloses several properties of the human sentrin-1 protein (SEQ ID NO:2) encoded by the open reading frame of SEQ ID NO:1, such as the ability to bind to Fas, TNFRI, or UBC9, the specification does provide sufficient guidance as to the nucleotide or amino acid sequences, or the physical or structural properties of any gene or protein, which comprises at least 100 nucleotides of SEQ ID NO:1 or 100 amino acids of SEQ ID NO:2 and which shares these properties. Further, the specification fails to provide guidance as to the amino acid residues which are critical to the observed biological activities of the protein corresponding to SEQ ID NO:2 such that amino acid sequences or nucleotide sequences which diverge from SEQ ID NOS: 1 or 2 and which encode a human sentrin-1 polypeptide can be determined from among the numerous possible nucleotide sequences which comprise a 100 nucleotide portion of SEQ ID NO:1. In particular, it is noted that SEQ ID NO:1, which is 1465 nucleotides, contains over 1100 nucleotides which are apparently non-coding sequence. The specification provides no guidance as to how any 100, or 200 nucleotide portion of the noncoding sequence of SEQ ID NO:1 can encode for a human

sentrin I polypeptide or provide any description of nucleic acid segments comprising 100-200 nucleotides of the non-coding sequence of SEQ ID NO:1 which are capable of inhibiting apoptosis. In addition, the claims read on nucleic acid segments which encode a human sentrin-1 polypeptide, wherein the segment encodes at least 100 contiguous amino acids of SEQ ID NO:2 and which is at least 85-95% identical to SEQ ID NO:2 (see claims 74-75). Since SEQ ID NO:2 consists of 101 amino acids, these claims read on human sentrin-1 polypeptides which are substantially larger than 101 amino acids. Neither the specification nor the prior art provides any teachings or description of amino acid sequences greater than 101 amino acids which encode a human sentrin-1 polypeptide. Other than SEQ ID NOS 1 and 2, the specification provides no description of any other nucleotide or amino acid sequences which encode human sentrin-1 or which inhibit apoptosis.

The previous office actions further stated that a gene is a chemical compound, albeit a complex one, and it is well established in our law that conception of a chemical compound requires that the inventor be able to define it so as to distinguish it from other materials, and to describe how to obtain it. See *Oka*, 849 F.2d at 583, 7 USPQ2d at 1171. Conception does not occur unless one has a mental picture of the structure of the chemical, or is able to define it by its method of preparation, its physical or chemical properties, or whatever characteristics sufficiently distinguish it. It is not sufficient to define it solely by its principal biological property, e.g., encoding human erythropoietin, because an alleged conception having no more specificity than that is simply a wish to know the identity of any material with that biological property. We hold

that when an inventor is unable to envision the detailed constitution of a gene so as to distinguish it from other materials, as well as a method for obtaining it, conception has not been achieved until reduction to practice has occurred. *Amgen Inc. v. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991). Further, *Vas-Cath Inc. V. Mahurkar*, 19USPQ2d 1111, clearly states that "applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention. The invention is, for purposes of 'written description' inquiry, whatever is claimed" (see page 1117). By failing to identify any nucleotide or amino acid sequence other than SEQ ID NOS: 1 or 2 which either encode a human sentrin-1 polypeptide or which have identical biological properties to the human sentrin-1, the specification does not "clearly allow persons or ordinary skill in the art to recognize that [he or she] invented what is claimed." (See *Vas-Cath* at page 1116). Adequate written description requires more than a mere statement that an element is part of the invention. Based on the applicant's specification, the skilled artisan cannot envision the detailed chemical structure of the encompassed nucleic acid sequences which may be capable of producing a human sentrin-1 polypeptide, and therefore conception is not achieved until reduction to practice has occurred, regardless of the complexity or simplicity of the method of isolation. See *Fiers v. Revel*, 25 USPQ2d 1602 at 1606 (CAFC 1993) and *Amgen Inc. V. Chugai Pharmaceutical Co. Ltd.*, 18 USPQ2d 1016. It was also noted that *Vas-Cath* makes clear that the written description provision of 35 U.S.C. 112 is severable from its enablement provision.

Therefore, based on the breadth of the claims as written and the lack of description in the specification for sequences which meet the claim limitations other than SEQ ID NOS 1 and 2, the specification fails to meet the burden for providing an adequate written description under 35 U.S.C. 112, first paragraph, for the subject matter encompassed by claims 73-75, 86-92, 94, and 96-10.

B. Claims 73-75, 85-92, and 94-101 stand rejected under 35 U.S.C. 112, first paragraph, for lack of enablement for the scope of the invention as claimed. This rejection is set forth below and in prior Office actions mailed on 9/28/01 and 6/5/02, Paper Nos. 10 and 13 respectively.

The previous office actions stated that the specification, while being enabling for a method of inhibiting Fas or TNFRI mediated apoptosis in cells *in vitro* comprising transfecting said cells with a nucleic acid expression construct encoding a nucleic acid comprising SEQ ID NO:1, does not reasonably provide enablement for methods of inhibiting any apoptotic pathway in cells in vitro or *in vivo* by administering any vector comprising or encoding any 100 nucleotide or amino acid portion of the nucleic acid or amino acid sequence of SEQ ID NO:1 or SEQ ID NO:2 respectively. The specification does not enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make or use the invention commensurate in scope with these claims.

This rejection comprises three (3) separate issues: **1)** the absence of an enabling disclosure for inhibiting any apoptotic pathway other than Fas or TNFRI in a cell *in vitro* or *in vivo* using applicant's claimed methods; **2)** the absence of an enabling disclosure for inhibiting Fas or TNFRI mediated apoptosis in a cell *in vitro* by contacting the cell with any nucleic acid segment other than SEQ ID NO:1; and **3)** the absence of an enabling disclosure for inhibiting Fas or TNFRI mediated apoptosis in a cell *in vivo* by contacting a cell with any nucleic acid comprising SEQ ID NO:1. These issues were identified by the Office after analysis of the disclosure provided by the specification. The Office has analyzed the specification in direct accordance to the factors outlined in *In re Wands*, namely 1) the nature of the invention, 2) the state of the prior art, 3) the predictability of the art, 4) the amount of direction or guidance present, and 5) the presence or absence of working examples, and presented detailed scientific reasons supported by publications from the prior art for the finding of a lack of enablement for the scope of the instant methods. The Wands analysis and supporting specific evidence are presented below for each of the identified issues.

As a first issue **(1)**, the specification does not provide an enabling disclosure for inhibiting any apoptotic pathway other than Fas or TNFRI in a cell *in vitro* or *in vivo* using Appellant's claimed methods. The broadest independent claim, claim 92, recites a method of inhibiting apoptosis in a cell comprising providing the cell with a nucleic acid segment comprising at least about 100 contiguous nucleotides of SEQ ID NO:1. Claims 94, and 96-101 depend on claim 92. Independent claim 73 recites a method of inhibiting apoptosis in a cell comprising providing the

cell with a nucleic acid segment encoding a human sentrin-1 polypeptide, wherein the segment encodes at least 100 contiguous amino acids of SEQ ID NO:2. The specification discloses a nucleotide sequence (SEQ ID NO:1) which is given the name human sentrin-1. The specification states that predicted human sentrin-1 polypeptide (SEQ ID NO:2) has homology to ubiquitin and other ubiquitin-like proteins, particularly to the yeast Smt3 protein. The specification further provides evidence that the protein product of SEQ ID NO:1 appears to localize to the nucleus and can bind to several different proteins including Fas and TNFRI, and that the transfection of a human cell line with plasmid encoding the full length SEQ ID NO:1 inhibits apoptosis in response to anti-Fas or TNF. The art at the time of filing teaches that several different apoptotic pathways exist in the cell which can be triggered by substantially different stimuli (Lavin et al. (1996) Experientia, Vol. 52, page 983, Figure 1; and Lieberthal et al. (1996) Am. J. Phys., Vol. 271 (3 part 2), page F483, column 1, paragraph 2). The specification teaches that while human sentrin-1 can bind to the death domains of Fas and TNFRI, it does not bind to CD40 or FADD/MORT1, proteins associated with other apoptotic pathways (specification, page 53, lines 25-29). While the specification does provide additional working examples which disclose the ability of sentrin-1 to bind to various other proteins, such as UBC9, ranGAP1 or PML, it does not provide any evidence that this binding results in the inhibition of apoptosis in a cell. UBC9, ranGAP1, and PML are not part of any known apoptotic pathways and the specification in fact suggests that sentrin interacts with these proteins in a process similar to ubiquitination. Neither the specification nor the art at the time of filing teaches that the process of protein ubiquitination results in the

inhibition of apoptosis. At the time of filing, it was well known that protein ubiquitination usually results in protein degradation. Thus, in view of the numerous different apoptotic pathways known at the time of filing, applicant's demonstration that sentrin-1 does not affect other apoptotic pathways such as FADD/MORT1 or CD40, the lack of correlation between the binding of sentrin to UBC9 , ranGAP1 or PML and any effect on apoptosis, and the breadth of the claims, the skilled artisan would have considered inhibiting any apoptotic pathway in a cell other than the FAS or TNFRI pathways by providing the cell with a nucleic acid encoding human sentrin-1 as highly unpredictable. As such it would have required undue experimentation to practice the scope of applicant's invention as claimed.

As a second issue **(2)**, the specification does not provide an enabling disclosure for inhibiting Fas or TNFRI mediated apoptosis in a cell *in vitro* by contacting the cell with any nucleic acid segment other than SEQ ID NO:1. The specification, as discussed above, provides evidence that the full length human sentrin -1 nucleic acid sequence encodes a polypeptide which when expressed in a murine or human cell is capable of inhibiting to a greater or lesser degree Fas or TNFRI mediated apoptosis. The claims as written, however, are substantially broader and read on the inhibition of apoptosis using nucleic acid segments which encode as little as 100 or 200 nucleotides of SEQ ID NO:1. The claims therefore read on the use of portions of SEQ ID NO:1 and also on unrelated sequences which contain 100 nucleotides of SEQ ID NO:1. The specification provides no guidance concerning any sequences which are not derived from SEQ ID NO:1 which comprise at least 100 nucleotides of SEQ ID NO:1 and which are capable of

inhibiting apoptosis in a cell. Further, the specification, while disclosing that portions of the nucleic acid sequence of a human sentrin or specifically of SEQ ID NO:1 can be used to inhibit apoptosis, does not provide sufficient guidance as to which portions, of the numerous possible nucleic acid sequences which may comprise 100 or more contiguous nucleotides of SEQ ID NO:1 or which may encode 200 or more contiguous amino acids of SEQ ID NO:2, retain the apoptosis inhibiting activity of the full length human sentrin -1 polypeptide, SEQ ID NO:2, encoded by SEQ ID NO:1. In regards to the nucleic acid sequence in SEQ ID NO:1, it is noted that over 1100 of the 1465 nucleotides listed, representing the first 90 and the last 1065 nucleotides of SEQ ID NO:1, do not apparently encode for any polypeptide (See Figure 2A). If any open reading frame exists in the first 90 nucleotides or the last 1065 nucleotides, the specification neither discloses the corresponding encoded amino acids or provide any guidance as to the nature or activity of any hypothetically encoded polypeptide. The specification further does not provide any guidance that the non-coding nucleotides of SEQ ID NO:1 have any anti-apoptotic activity. As the specification teaches that the anti-apoptotic activity of the human sentrin 1 polypeptide comprising the amino acid sequence of SEQ ID NO:2 is the result of protein:protein interactions, the skilled artisan would have considered it highly unpredictable whether any 100 or 200 contiguous nucleotides from the non-coding portion of SEQ ID NO:1 would have any effect on apoptosis in a cell. In addition, Figure 1A clearly demonstrates that only the **full length** human sentrin-1 corresponding to the entire 101 amino acids of SEQ ID NO:2 can bind to the Fas death domain. Fragments consisting of 1-23AA, 1-70AA, and 24-97AA of SEQ ID NO:2 failed to exhibit Fas binding

(specification, Figure 1A). In the absence of specific guidance from the specification and in view of the results depicted in Figure 1, it is unclear which residues are essential for Fas or TNFRI binding and apoptosis inhibition and thus, the skilled artisan would not be able to predict whether any portion of the amino acid sequence of SEQ ID NO:2 would be capable of retaining the apoptosis inhibiting activity of the full length human sentrin-1 protein. Further, as discussed in the previous paragraph, while the specification provides data concerning the binding of sentrin-1 to various proteins other than Fas or TNFRI and provides some analysis of sentrin domains required for binding to UBC9 , ranGAP1 or PML, the specification has not related the "sentrinization" of these proteins with any effect on apoptosis. Thus, in view of the failure of the 1-70AA, 24-97AA, and 1-23AA portions of sentrin-1 to bind to Fas, the lack of guidance concerning specific portions of the sentrin polypeptide which retain Fas and/or TNFRI binding and anti-apoptotic activity, the lack of correlation between applicant's binding studies of sentrin to UBC9 , ranGAP1 or PML and any effects on apoptosis or sentrin binding to Fas or TNFRI, and the breadth of the claims, the skilled artisan not have been able to predict without undue experimentation whether any 100 nucleotide portion of SEQ ID NO:1 or a nucleic acid sequence encoding any 100 amino acid portion of SEQ ID NO:2 would be capable of inhibiting Fas or TNFRI mediated apoptosis.

As a third issue **(3)**, the specification does not provide an enabling disclosure for inhibiting Fas or TNFRI mediated apoptosis in a cell *in vivo* by contacting a cell with any nucleic acid comprising SEQ ID NO:1. Claims 73-75, 85-89, 92, and 94-99 are broad and read on any type of nucleic acid segment. Claims 90 and 100 recite wherein the nucleic acid segment is operatively

linked to a promoter and claims 91 and 101 recite wherein the nucleic acid segment is operatively linked to a promoter and comprised within a vector. Thus, the broadest claims read on nucleic acid segments which are not expressible as they are not linked to a promoter or other transcriptional regulatory elements. As noted above, the specification teaches that the anti-apoptotic activity of the human sentrin 1 polypeptide comprising the amino acid sequence of SEQ ID NO:2 is the result of protein:protein interactions. The specification only provides guidance for the expression of sentrin-1 using vectors wherein the sentrin-1 is operatively linked to a promoter. Regarding the expression of sentrin-1 *in vivo*, the specification discloses that a pharmaceutical composition comprising a nucleic acid encoding sentrin-1 can be administered to a mammal in order to inhibit apoptosis. The specification does not disclose any disease or condition associated with apoptosis. Further, the specification fails to provide any guidance concerning the characteristics of cells to be targeted for apoptosis inhibition, the level of sentrin expression from any vector in such a target cell which correlates with apoptosis inhibition, the routes and dosages of administration of any vector encoding sentrin to a mammal such that the target cells or organs are transfected, or the level and duration of apoptosis inhibition within a target cell population which correlates with any effect on any symptom of an apoptosis related disease or condition. At the time of filing, in vivo gene therapy utilizing the direct administration of recombinant nucleic acids, whether in the form of retroviruses, adenoviruses, or adeno-associated viruses, was considered to be highly unpredictable. Verma et al. states that, "[t]he Achilles heel of gene therapy is gene delivery..", and that, "most of the approaches suffer

from poor efficiency of delivery and transient expression of the gene" (Verma et al. (1997) Science, Vol. 389, page 239, column 3, paragraph 2). Marshall concurs, stating that, ".. difficulties in getting genes transferred efficiently to target cells- and getting them expressed- remain a nagging problem for the entire field", and that, "many problems must be solved before gene therapy will be useful for more than the rare application" (Marshall (1995) Science, Vol. 269, page 1054, column 3, paragraph 2, and page 1055, column 1). Orkin et al. further states in a report to the NIH that, " .. none of the available vector systems is entirely satisfactory, and many of the perceived advantages of vector systems have not been experimentally validated", and that," [w]hile the expectations and the promise of gene therapy are great, clinical efficacy has not been definitively demonstrated at this time in any gene therapy protocol" (Orkin et al. (1995) "Report and recommendations of the panel to assess the NIH investment in research on gene therapy", page 1, paragraph 3, and page 8, paragraph 2). Among the many factors that the art teaches affect efficient gene delivery and sustained gene expression are anti-viral immune responses, and the identity of the promoter used to drive gene expression. Thus, the art at the time of filing clearly establishes that the expectation for achieving a desired therapeutic effect in vivo by expressing a therapeutic gene using any of the expression constructs known in the art at the time of filing was extremely low. Therefore, in view of the art recognized high level of unpredictability in treating disease using recombinant vectors at the time of filing, the lack of guidance provided by the specification for the parameters affecting vector delivery and gene expression in vivo, the lack of correlation between applicant's in vitro working examples and the therapeutic inhibition of

apoptosis in a mammal, and the breadth of the claims, it would have required undue experimentation to practice the scope of the invention as claimed.

(11) Detailed Rebuttal to Appellant's arguments

A. Regarding the rejection of claims 73-75, 86-92, 94, and 96-101 for lack of written description, the appellant argues that possession of the instant invention has been demonstrated by disclosure of the full-length sequence of the human sentrin polypeptide (SEQ ID NO:2) and its underlying cDNA sequence (SEQ ID NO:1), citing *Lockwood v. Am. Airlines, Inc.* The appellant further argues that based on the disclosure of SEQ ID NO:1 and SEQ ID NO:2, portions of the sentrin polypeptide comprising 20, 30, 40 up to 100 contiguous amino acid residues from SEQ ID NO:2 and their corresponding nucleotide sequences can be ascertained.

In response, it has been noted that appellant's claims are not limited to the full-length sequence of SEQ ID NO:1 or SEQ ID NO:2. Nor are the claims limited to nucleic acid segments which comprise all or part of the coding region of human sentrin-1. The broadest independent claim is claim 92 which recites a method of inhibiting apoptosis in a cell comprising providing the cell with a nucleic acid segment comprising at least about 100 contiguous nucleotides of SEQ ID NO:1. At its broadest interpretation, this claim reads on nucleic acids which share no similarity whatsoever with SEQ ID NO:1 except for 100 contiguous nucleotides. Claim 73 recites a method

of inhibiting apoptosis in a cell comprising providing the cell with a nucleic acid segment encoding a human sentrin-1 polypeptide, wherein the segment encodes at least 100 contiguous amino acids of SEQ ID NO:2. Further, the claims, as exemplified by claims 88 and 98 recite wherein the nucleic acid segment is effective to prevent apoptosis in a cell. The specification fails to identify or describe any nucleic acid, gene, or cDNA which comprises 100 or more contiguous nucleotides of SEQ ID NO:1 and which encodes a human sentrin-1 or a protein with properties similar to human sentrin-1 and capable of inhibiting apoptosis in a cell. In addition, the claims 74-75 which depend on claim 73 read on nucleic acid segments which encode a human sentrin-1 polypeptide, wherein the segment encodes at least 100 contiguous amino acids of SEQ ID NO:2 and which is at least 85-95% identical to SEQ ID NO:2 (see claims 74-75). Since SEQ ID NO:2 consists of 101 amino acids, these claims read on human sentrin-1 polypeptides which are substantially larger than 101 amino acids. The specification does not provide a description of the properties or sequence of any nucleic acid which encodes human sentrin-1 other than SEQ ID NO:1. Further, while the specification does disclose several properties of the human sentrin-1 protein (SEQ ID NO:2) encoded by the open reading frame of SEQ ID NO:1, such as the ability to bind to Fas, TNFRI, or UBC9, the specification does provide sufficient guidance as to the nucleotide or amino acid sequences, or the physical or structural properties of any gene or protein, which comprises at least 100 nucleotides of SEQ ID NO:1 or 100 amino acids of SEQ ID NO:2 and which shares these properties. Further, the specification fails to provide guidance as to the amino acid residues which are critical to the observed biological activities of the protein corresponding to SEQ ID NO:2

such that amino acid sequences or nucleotide sequences which diverge from SEQ ID NOS: 1 or 2 and which encode a human sentrin-1 polypeptide can be determined from among the numerous possible nucleotide sequences which comprise a 100 nucleotide portion of SEQ ID NO:1 or which encode 100 amino acids of SEQ ID NO:2. Thus, applicant's disclosure of a single nucleotide sequence, SEQ ID NO:1 and a single amino acid sequence, SEQ ID NO:2 is not commensurate in scope with the numerous nucleic acid segments encompassed by the claims.

The appellant further provides several polypeptide sequences which the appellant states could be determined from the description provided in the specification. The amino acid sequences listed appear to be derived from SEQ ID NO:2, the putative translation product of the open reading frame of SEQ ID NO:1. None of these particular segments are specifically disclosed in the specification. The specification generally states that at least 10-20, 20-30, 50-60, or up to 70, 80, 90, or 100 contiguous amino acid residues can be used in the instant methods. However, as discussed in previous office action and in section (10) above, the specification fails to disclose or describe by physical or chemical properties which segments of SEQ ID NO:2 or SEQ ID NO:1 are capable of inhibiting apoptosis in a cell. The appellant does not believe that this issue is relevant for a written description rejection. However, the claims as written are drawn to methods of inhibiting apoptosis, and claims 88 and 98 clearly recite the limitation that the nucleic acid segment is effective to prevent apoptosis in a cell. Therefore, as noted by appellants, in order to show possession of the instant invention at the time of filing, the specification is required to provide a written description that **fully** sets forth the claimed invention by "such descriptive

means as words, structures, figures, diagrams, formulas, etc.,". *Lockwood v. Am. Airlines*. The specification only provides a description of SEQ ID NO:1 which is capable of inhibiting apoptosis. The specification fails to provide sufficient description of any other nucleic acid segment that meets the claim limitations.

Regarding various citations of case law presented in papers no. 10 and 13, the appellant states that *Amgen Inc. v. Chugai Pharmaceuticals Co., Ltd.* and *Fiers V. Revel* are not applicable to the present issue. The office disagrees. The *Amgen* decision is on point in that the court in *Amgen* determined that the specification failed to provide an adequate written description of claims that encompassed numerous nucleic acid sequences with the biological activity of EPO whereas the specification only provided a single EPO sequence and limited description of any analogs. In the instant case, the claims read on numerous different nucleic acid segments with the activity of inhibiting apoptosis whereas the specification only discloses full length SEQ ID NO:1. *Fiers v. Revel* is also on point in this case. In *Fiers*, the court determined claims which cover all DNAs that code for a protein are analogous to a single means claim, which has been held not to comply with the first paragraph of section 112. See *In re Hyatt*, 708 F.2d 712,218 USPQ 195, 197 (Fed. Cir. 1983) ("the enabling disclosure of the specification [must] be commensurate in scope with the claim under consideration.") Claiming all DNA's that achieve a result without defining what means will do so is not in compliance with the description requirement; it is an attempt to preempt the future before it has arrived. The instant claims, particularly claims 92, 94, and 96-101 read on nucleic acid segments which are encompass any nucleic acid which comprises

100 contiguous nucleotides of SEQ ID NO:1 and which can inhibit apoptosis in a cell. The specification only discloses a single nucleic acid, SEQ ID NO:1 which meets the claim limitations. Thus, the specification does provide sufficient written description of the claims as written to satisfy the requirements of 35 U.S.C. 112, first paragraph.

B. Regarding the rejection of claims 73-75, 85-92, and 94-101 for scope of enablement, the appellant states that the standard for enablement is that, "the specification must teach those skilled in the art how to make and use the full scope of the claimed invention without 'undue experimentation'.", citing *In re Wright*. The appellant argues that the specification enables the full scope of the invention by demonstrating the inhibition of Fas or TNFRI mediated apoptosis *in vitro* by contacting cells with an expression construct comprising SEQ ID NO:1. The appellant argues that there is a reasonable correlation between the subject matter indicated as enabled by the Office and the scope of the claims as written, and that the Office has not presented specific evidence why the specification does not enable the claims as written, citing *In re Marzochhi*. The appellant further states that, "as long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied", citing *In re Fisher* and MPEP 2164.01(b).

In response, the office action has in fact analyzed the specification for enablement of the claimed invention in direct accordance to the factors outlined in *In re Wands*, namely 1) the nature

of the invention, 2) the state of the prior art, 3) the predictability of the art, 4) the amount of direction or guidance present, and 5) the presence or absence of working examples, and presented detailed scientific reasons supported by publications from the art for the finding of a lack of enablement for the scope of the instant methods, see section (10) above and papers no. 10 and 13. Please note that case law including the Marzocchi decision sanctions both the use of sound scientific reasoning and printed publications to support a holding of non-enablement (see *In re Marzocchi* 169 USPQ 367, and *Ex parte Sudilovsky* 21 USPQ2d 1702). As noted by appellants, 35 U.S.C. 112 requires that the scope of the claims must bear a reasonable correlation to the scope of enablement provided by the specification to persons of ordinary skill in the art. *In re Fisher*, 166 USPQ 18, 24 (CCPA 1970). Based on the Wands analysis of the instant specification, see section (10) above, the office finds that the scope of the instant claims does not bear a reasonable correlation to the scope of enablement provided by the specification and as such does not meet the requirements of 35 U.S.C. 112, first paragraph. As discussed in detail in section (10) above, the office has identified three (3) issues which are not enabled by the instant specification: **1)** the absence of an enabling disclosure for inhibiting any apoptotic pathway other than Fas or TNFRI in a cell *in vitro* or *in vivo* using applicant's claimed methods; **2)** the absence of an enabling disclosure for inhibiting Fas or TNFRI mediated apoptosis in a cell *in vitro* by contacting the cell with any nucleic acid segment other than SEQ ID NO:1; and **3)** the absence of an enabling disclosure for inhibiting Fas or TNFRI mediated apoptosis in a cell *in vivo* by contacting a cell

with any nucleic acid comprising SEQ ID NO:1. Applicant's specific arguments as they pertain to each of these issues are addressed below.

Regarding issue **1)**, the absence of an enabling disclosure for inhibiting any apoptotic pathway other than Fas or TNFRI in a cell *in vitro* or *in vivo* using applicant's claimed methods, the appellant states that the office has not provided any evidence of why sentrin would not achieve inhibition of any apoptotic pathway and further that the specification provides a demonstration of inhibition of the Fas and TNFRI pathways of apoptosis. Contrary to appellant's statements, the office has in fact provided specific evidence and arguments why the skilled artisan would have doubted the ability of human sentrin-1 to inhibit any pathway of apoptosis. The office cited the teachings of Lavin et al. and Lieberthal et al. that many different apoptotic pathways exist in the cell and that substantially different signals are involved in the different pathways. The office also provided evidence from the appellant's own specification that human sentrin-1 (SEQ ID NO:1) does not appear to affect CD40 and FADD/MORT1 associated apoptotic pathways, see the specification at page 53, lines 25-29. The office also discussed the fact that while the specification does provide additional working examples which disclose the ability of sentrin-1 to bind to various other proteins, such as UBC9, ranGAP1 or PML, it does not provide any evidence that this binding results in the inhibition of apoptosis in a cell. UBC9, ranGAP1, and PML are not part of any known apoptotic pathways and the specification in fact suggests that sentrin interacts with these proteins in a process similar to ubiquitination. Neither the specification nor the art at the time of filing teaches that the process of protein ubiquitination results in the

inhibition of apoptosis. At the time of filing, it was well known that protein ubiquitination usually results in protein degradation. Thus, the office concluded that in view of the numerous different apoptotic pathways known at the time of filing, applicant's demonstration that sentrin-1 does not affect other apoptotic pathways such as FADD/MORT1 or CD40, the lack of correlation between the binding of sentrin to UBC9 , ranGAP1 or PML and any effect on apoptosis, and the breadth of the claims, the skilled artisan would have considered inhibiting any apoptotic pathway in a cell other than the FAS or TNFRI pathways by providing the cell with a nucleic acid encoding human sentrin-1 as highly unpredictable. As such it would have required undue experimentation to practice the scope of applicant's invention as claimed. The appellants have not provided specific arguments which refute the evidence for non-enablement provided by the office.

Regarding issue **2),** the absence of an enabling disclosure for inhibiting Fas or TNFRI mediated apoptosis in a cell *in vitro* by contacting the cell with any nucleic acid segment other than SEQ ID NO:1, the appellant argues that Figure 1 shows different sentrin constructs and that there is no reason why it would require undue experimentation to make and use such nucleic acid segments. In response, the office has previously noted that there are two broad independent claims in the claims on appeal. The broadest independent claim, claim 92, recites a method of inhibiting apoptosis in a cell comprising providing the cell with a nucleic acid segment comprising at least about 100 contiguous nucleotides of SEQ ID NO:1. Claims 94, and 96-101 depend on claim 92. Independent claim 73 recites a method of inhibiting apoptosis in a cell comprising providing the cell with a nucleic acid segment encoding a human sentrin-1 polypeptide, wherein

the segment encodes at least 100 contiguous amino acids of SEQ ID NO:2. Claims 74-75, and 85-91 depend on claim 73. Figure 1 depicts several fragments of SEQ ID NO:1. However, all of the fragments depicted comprise part of the open reading frame that translates into SEQ ID NO:2. As discussed above, claims 92, 94, and 96-101 are not so limited and read on the inhibition of apoptosis using nucleic acid segments which encode as little as 100 or 200 nucleotides of SEQ ID NO:1. SEQ ID NO:1 consists of 1465 nucleotides of which over 1100 do not apparently encode for any polypeptide (See Figure 2A). If any open reading frame exists in the first 90 nucleotides or the last 1065 nucleotides, the specification neither discloses the corresponding encoded amino acids or provide any guidance as to the nature or activity of any hypothetically encoded polypeptide. The specification further does not provide any guidance that the non-coding nucleotides of SEQ ID NO:1 have any anti-apoptotic activity. As the specification teaches that the anti-apoptotic activity of the human sentrin 1 polypeptide comprising the amino acid sequence of SEQ ID NO:2 is the result of protein:protein interactions, the skilled artisan would have considered it highly unpredictable whether any 100 or 200 contiguous nucleotides from the non-coding portion of SEQ ID NO:1 would have any effect on apoptosis in a cell. In addition, Figure 1A clearly demonstrates that only the **full length** human sentrin-1 corresponding to the entire 101 amino acids of SEQ ID NO:2 can bind to the Fas death domain. Fragments consisting of 1-23AA, 1-70AA, and 24-97AA of SEQ ID NO:2 failed to exhibit Fas binding (specification, Figure 1A). In the absence of specific guidance from the specification and in view of the results depicted in Figure 1, it is unclear which residues are essential for Fas or TNFRI binding and

apoptosis inhibition and thus, the skilled artisan would not be able to predict whether any portion of the amino acid sequence of SEQ ID NO:2 would be capable of retaining the apoptosis inhibiting activity of the full length human sentrin-1 protein. Further, as discussed in the previous paragraph, while the specification provides data concerning the binding of sentrin-1 to various proteins other than Fas or TNFRI and provides some analysis of sentrin domains required for binding to UBC9 , ranGAP1 or PML, the specification has not related the "sentrinization" of these proteins with any effect on apoptosis. In addition, the claims read on the use of unrelated sequences which contain 100 nucleotides of SEQ ID NO:1. The specification provides no guidance concerning any sequences which are not derived from SEQ ID NO:1 which comprise at least 100 nucleotides of SEQ ID NO:1 and which are capable of inhibiting apoptosis in a cell. Further, the specification, while disclosing that portions of the nucleic acid sequence of a human sentrin or specifically of SEQ ID NO:1 can be used to inhibit apoptosis, does not provide sufficient guidance as to which portions, of the numerous possible nucleic acid sequences which may comprise 100 or more contiguous nucleotides of SEQ ID NO:1 or which may encode 100 or more contiguous amino acids of SEQ ID NO:2, retain the apoptosis inhibiting activity of the full length human sentrin -1 polypeptide, SEQ ID NO:2, encoded by SEQ ID NO:1. Thus, in view of the failure of the 1-70AA, 24-97AA, and 1-23AA portions of sentrin-1 to bind to Fas, the lack of guidance concerning specific portions of the sentrin polypeptide which retain Fas and/or TNFRI binding and anti-apoptotic activity, the lack of correlation between applicant's binding studies of sentrin to UBC9 , ranGAP1 or PML and any effects on apoptosis or sentrin binding to Fas or

TNFRI, and the breadth of the claims, the skilled artisan not have been able to predict without undue experimentation whether any 100 nucleotide portion of SEQ ID NO:1 or a nucleic acid sequence encoding any 100 amino acid portion of SEQ ID NO:2 would be capable of inhibiting Fas or TNFRI mediated apoptosis. Finally, please note that the grounds of rejection identified herein as issue **2)** does not pertain and has not been applied to claims 85 and 95. Claims 85 and 95 are rejected for lack of enablement based on the grounds of rejection identified herein as issues **1)** and **3)**.

Regarding issue **3),** the absence of an enabling disclosure for inhibiting Fas or TNFRI mediated apoptosis in a cell *in vivo* by contacting a cell with any nucleic acid comprising SEQ ID NO:1, the appellant argues that the Orkin article cited by the office in fact teaches that more than 100 protocols involving gene therapy have been approved and there have been claims of successful gene therapy. The appellants also argue that, "No doubt each of these protocols was preceded by *in vitro* data", appeal brief, page 8. The appellant also states that a recent NIH report states that 80 clinical trials with adenovirus vector were initiated since 1993. Based on these statements, the appellant concludes that the office is incorrect in stating that the skilled artisan would have considered therapeutic gene expression using gene therapy unpredictable at the time of filing. In response, the office is unclear which "recent NIH report" the appellant is referring to on page 9, first paragraph. The Orkin et al. report, which is an NIH report, was written in December 1995. Since the effective filing date of the instant application is 11/5/96, Orkin et al. represents the state of the art at the time of filing. Further, Orkin et al., while disclosing that more

than 100 gene therapy protocols have been approved for phase one clinical trial, does not conclude that the technique of gene therapy is predictable or that *in vitro* results correlate with *in vivo* success. In fact, Orkin et al. actually states on page 1 of the report, which summarizes the findings of the Panel on Gene Therapy, that, " [w]hile the expectations and the promise of gene therapy are great, clinical efficacy has not been definitively demonstrated at this time in any gene therapy protocol despite anecdotal claims of successful therapy and the initiation of more than 100 Recombinant DNA advisory Committee (RAC)-approved protocols" (Orkin et al., page 1). Orkin goes on to state that, "[s]ignificant problems remain in all basic aspects of gene therapy. Major difficulties at the basic level include shortcomings in all current gene transfer vectors and an inadequate understanding of the biological interaction of these vectors with the host" (Orkin et al., page 1). Furthermore, contrary to appellants belief, Orkin does not support the concept that *in vitro* data provides a reasonable correlation to *in vivo* success. Orkin et al. in fact states that even *in vivo* animal studies are not always correlative to clinical efficacy (Orkin et al., pages 2 and 14). Furthermore, case law has also recognized that *in vitro* results do not necessarily correlate with *in vivo* efficacy. *Ex parte Maas*, 9 USP2d 1746, states, "First, although appellant's specification describes certain *in vitro* experiments, there is no correlation on this record between *in vitro* experiments and a practical utility in currently available form for humans or animals. It is not enough to rely on *in vitro* studies where, as here, a person having ordinary skill in the art has no basis for perceiving those studies as constituting recognized screening procedures with clear relevance to utility in humans or animals". In addition, the instant grounds of rejection are not

based on the "unpredictability of gene therapy" *per se*, but rather on the unpredictability of achieving therapeutic expression of a target gene using expression systems and vectors currently available at the time of filing. As discussed in papers 10 and 13 and in section (10) above, claims 73-75, 85-89, 92, and 94-99 are broad and read on the use of any type of nucleic acid segment to inhibit apoptosis in cells. Claims 90 and 100 recite wherein the nucleic acid segment is operatively linked to a promoter and claims 91 and 101 recite wherein the nucleic acid segment is operatively linked to a promoter and comprised within a vector. Thus, the broadest claims read on nucleic acid segments which are not expressible as they are not linked to a promoter or other transcriptional regulatory elements. As noted above, the specification teaches that the anti-apoptotic activity of the human sentrin 1 polypeptide comprising the amino acid sequence of SEQ ID NO:2 is the result of protein:protein interactions. The specification only provides guidance for the expression of sentrin-1 using vectors wherein the sentrin-1 is operatively linked to a promoter. Regarding the expression of sentrin-1 *in vivo*, the specification discloses that a pharmaceutical composition comprising a nucleic acid encoding sentrin-1 can be administered to a mammal in order to inhibit apoptosis. The specification does not disclose any disease or condition associated with apoptosis. Further, the specification fails to provide any guidance concerning the characteristics of cells to be targeted for apoptosis inhibition, the level of sentrin expression from any vector in such a target cell which correlates with apoptosis inhibition, the routes and dosages of administration of any vector encoding sentrin to a mammal such that the target cells or organs are transfected, or the level and duration of apoptosis inhibition within a

target cell population which correlates with any effect on any symptom of an apoptosis related disease or condition. At the time of filing, expression of therapeutic levels of target genes, utilizing the direct administration of recombinant nucleic acids, whether in the form of retroviruses, adenoviruses, or adeno-associated viruses, was considered to be highly unpredictable. Verma et al. was cited for teaching that, "[t]he Achilles heel of gene therapy is gene delivery..", and that, "most of the approaches suffer from poor efficiency of delivery and transient expression of the gene" (Verma et al. (1997) Science, Vol. 389, page 239, column 3, paragraph 2). Marshall was also cited for stating that, ".. difficulties in getting genes transferred efficiently to target cells- and getting them expressed- remain a nagging problem for the entire field", and that, "many problems must be solved before gene therapy will be useful for more than the rare application" (Marshall (1995) Science, Vol. 269, page 1054, column 3, paragraph 2, and page 1055, column 1). Finally, Orkin et al. was cited for teaching that " .. none of the available vector systems is entirely satisfactory, and many of the perceived advantages of vector systems have not been experimentally validated", and that," [w]hile the expectations and the promise of gene therapy are great, clinical efficacy has not been definitively demonstrated at this time in any gene therapy protocol" (Orkin et al. (1995) "Report and recommendations of the panel to assess the NIH investment in research on gene therapy", page 1, paragraph 3, and page 8, paragraph 2). Among the many factors that the cited art teaches affect efficient gene delivery and sustained gene expression are anti-viral immune responses, and the identity of the promoter used to drive gene expression. Thus, the art at the time of filing clearly establishes that the expectation for achieving

a desired therapeutic effect *in vivo* by expressing a therapeutic gene using any of the expression constructs known in the art at the time of filing was extremely low. Therefore, in view of the art recognized high level of unpredictability in treating disease using recombinant vectors at the time of filing, the lack of guidance provided by the specification for the parameters affecting vector delivery and gene expression *in vivo*, the lack of correlation between applicant's *in vitro* working examples and the therapeutic inhibition of apoptosis in a mammal, and the breadth of the claims, it would have required undue experimentation to practice the scope of the invention as claimed. It is noted that the applicant has not provided any specific arguments regarding the teachings of Marshall or Verma or provided any arguments or evidence regarding the lack of enablement for the scope of nucleic acids, i.e. viral vectors, plasmid, etc., encompassed by the claims.

In conclusion, the Office has analyzed the specification in direct accordance with the guidelines established in the MPEP and in direct accordance with the factors outlined in *In re Wands*, namely 1) the nature of the invention, 2) the state of the prior art, 3) the predictability of the art, 4) the amount of direction or guidance present, and 5) the presence or absence of working examples, and presented detailed scientific reasons supported by publications from the art for the finding of a lack of enablement for the scope of the instant methods. Thus, the Office has met its burden in providing substantial and specific evidence that the specification does not enable the scope of the claims as written.

For the above reasons, it is believed that the rejections should be sustained.

Respectfully submitted,

A.M.S. Wehbé, Ph.D.

July 14, 2003

ANNE M. WEHBE' PH.D
PRIMARY EXAMINER

Committee members:

Deborah Reynolds
Michael Woodward

DJR

DEBORAH J. REYNOLDS
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

MICHAEL P. WOODWARD
SUPERVISORY PATENT EXAMINER
TECHNOLOGY CENTER 1600

CONFEREE

A Novel Ubiquitin-like Modification Modulates the Partitioning of the Ran-GTPase–activating Protein RanGAP1 between the Cytosol and the Nuclear Pore Complex

Michael J. Matunis, Elias Coutavas, and Günter Blobel

Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, New York 10021

Abstract. Ran is a nuclear Ras-like GTPase that is required for the bidirectional transport of proteins and ribonucleoproteins across the nuclear pore complex (NPC). A key regulator of the Ran GTP/GDP cycle is the 70-kD Ran-GTPase–activating protein RanGAP1. Here, we report the identification and localization of a novel form of RanGAP1. Using peptide sequence analysis and specific mAbs, RanGAP1 was found to be modified by conjugation to a ubiquitin-like protein. Immunoblot analysis and immunolocalization by light and EM demonstrated that the 70-kD unmodified form of RanGAP1 is exclusively cytoplasmic, whereas the 90-kD modified form of RanGAP1 is associated with the cytoplasmic fibers of the NPC. The modified form of RanGAP1 also appeared to associate with the mitotic spindle apparatus during mitosis. These findings have specific implications for Ran function and broad implications for protein regulation by ubiquitin-like modifications. Moreover, the variety and function of ubiquitin-like protein modifications in the cell may be more diverse than previously realized.

TRANSPORT of macromolecules across the nuclear envelope occurs bidirectionally through nuclear pore complexes (NPCs)[1], large supramolecular assemblies that span both membranes of the nuclear envelope (Rout and Wente, 1994). Whereas small ions and metabolites can passively diffuse through the NPC, most proteins and ribonucleoproteins are transported across the NPC by a signal- and energy-dependent mechanism. Dissection of the events culminating in nuclear import has been aided by the development of a permeabilized cell system that has made possible the identification of soluble cytosolic factors required for nuclear import (Adam et al., 1990), and more recently by the development of solution binding assays that use recombinant transport factors and nucleoporins (Rexach and Blobel, 1995).

Using the permeabilized cell assay in conjunction with biochemical fractionation of cytosolic extracts, four factors required for nuclear import have been purified and characterized (Moore and Blobel, 1993, 1994; Melchior et al., 1993; Adam and Adam, 1994; Görlich et al., 1994, 1995; Chi et al., 1995: Enenkel et al., 1995; Imamoto et al., 1995; Moroianu et al., 1995*a*: Paschal and Gerace, 1995; Radu et al., 1995). Recognition of nuclear localization signal (NLS)-containing substrates in the cytoplasm is mediated by the α subunit of the karyopherin-α/β heterodimer (Enenkel et al., 1995; Moroianu et al., 1995*a*; Weis et al., 1995), and docking of the trimeric complex to the NPC is mediated by the β subunit (Moroianu et al., 1995*b*; Radu et al., 1995). Peptide repeat–containing nucleoporins (Rout and Wente, 1994), which bind karyopherin-β in vitro and are components of the cytoplasmic and nucleoplasmic fibers as well as the peripheries of the central channel, have been proposed to form an array of substrate/receptor docking sites (Radu et al., 1995). The small GTPase Ran and the Ran-interactive protein p10 are believed to mediate the dissociation of substrate/receptor complexes as they dock along these sites (Melchior et al., 1993; Moore and Blobel, 1993, 1994; Paschal and Gerace, 1995). While the exact nature and molecular dynamics of translocation across the NPC remain to be elucidated, in vitro binding assays have recently revealed functional relationships among the karyopherin heterodimer, Ran, p10, and peptide repeat-containing nucleoporins. p10 binds directly to the peptide repeat–containing nucleoporins, Ran-GDP, and karyopherin-β, and has been proposed to coordinate substrate/receptor dissociation reactions at the NPC (Nehrbass and Blobel, 1996; Paschal et al., 1996; Matunis, M.J., G. Blobel, and M. Hijikata, unpublished data). Actual release of docked substrate/receptor complexes appears to be mediated by the

Address all correspondence to Günter Blobel, Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, 1230 York Avenue, New York, NY 10021. Tel.: (212) 327-8096; Fax: (212) 327-7880; E-mail: Blobel@rockvax.rockefeller.edu

1. *Abbreviations used in this paper*: BRL, buffalo rat liver (cells); EST, expressed sequence tagged; NEM, *N*-ethylmaleimide; NPC, nuclear pore complex; PCLF, pore complex lamina formation.

© The Rockefeller University Press, 0021-9525/96/12/1457/14 $2.00

binding of Ran-GTP to karyopherin-β, which dissociates the karyopherin heterodimer (Rexach and Blobel, 1995; Moroianu et al., 1996). All these findings point to Ran and factors regulating its guanine nucleotide state as key determinants of nucleocytoplasmic transport.

Similar to other GTPases, Ran is presumed to function as a molecular switch, associating with and dissociating from interacting proteins through conformational changes driven by GTP hydrolysis or by nucleotide exchange (Bourne et al., 1991; Rush et al., 1996; Sazer, 1996). The intrinsic rates of hydrolysis and exchange by Ran are extremely low, and these reactions are therefore catalyzed by regulatory factors whose subcellular distributions define Ran's activities. Nucleotide exchange by Ran is catalyzed by the GDP–GTP exchange factor RCC1 (Bischoff and Ponstingl, 1991), and GTP hydrolysis is catyalyzed by the GTPase-activating protein RanGAP1 (Bischoff et al., 1994, 1995*a*,*b*). RCC1 is a nuclear chromatin–associated protein, initially identified as a factor involved in the control of mitotic events, including chromatin condensation and cell cycle progression (Ohtsubo et al., 1987, 1989; Bischoff et al., 1990; Dasso, 1993). RanGAP1, on the other hand, was initially identified in *Saccharomyces cerevisiae* as *RNA1-1*, a mutant defective in RNA production, processing, and nuclear export (Hartwell, 1967; Traglia et al., 1989). Immunolocalization of Rna1p identified it as a cytoplasmic protein that is excluded from the nucleus (Hopper et al., 1990). The mammalian RanGAP1 was purified from HeLa cells as a homodimer of 65-kD subunits that specifically enhanced the rate of Ran-GTP hydrolysis by three orders of magnitude (Bischoff et al., 1994, 1995*a*,*b*). In general, these findings imply that Ran is converted to its GTP-bound form in the nucleus and hydrolyzed to its GDP-bound form in the cytoplasm. Two additional proteins that bind Ran-GTP and likely effect GTP hydrolysis are the cytosolic factor RanBP1 and the nucleoporin Nup358 (Coutavas et al., 1993; Wu et al., 1995; Yokoyama et al., 1995). RanBP1 and Nup358 contain domains that have been reported to enhance RanGAP1-induced Ran-GTP hydrolysis by an order of magnitude (Bischoff et al., 1995*b*; Beddow et al., 1995). The localization of Nup358 to the cytoplasmic fibers of the NPC suggests that an early step in nuclear import may involve GTP hydrolysis at or near these fibers. Consistent with this possibility, several reports have suggested a concentration of RanGAP1 at the nuclear envelope (Melchior et al., 1993; Bischoff et al., 1994; Koepp et al., 1996).

While the subcellular distributions of RanGAP1 and RCC1 seem to be strictly defined based on immunofluorescence localization and cell fractionation, it remains to be determined whether these factors are more precisely localized at the site of nuclear transport (i.e., the NPC). Targeting of a subpopulation of RCC1 and/or RanGAP1 to the NPC could potentially be regulated by modification of these factors. One modification that specifies protein targeting is ubiquitination, which is a posttranslational modification involving the covalent ligation of the carboxyl terminus of ubiquitin to internal lysine residues in a host of intracellular proteins (Hershko and Ciechanover, 1992; Wilkinson, 1995). Whereas the primary fate of ubiquitinated proteins is degradation by the 26S proteosome (Ciechanover, 1994; Jentsch and Schlenker, 1995), it has long been recognized that ubiquitination is likely to have roles beyond proteolysis. Ubiquitin conjugation has been recently shown to act as a signal for endocytosis and vacuolar targeting (Egner and Kuchler, 1996; Galan et al., 1996; Hicke and Riezman, 1996; Roth and Davis, 1996; Strous et al., 1996) and as regulator of IκB kinase activity (Chen et al., 1996). In addition to functions other than proteolysis, a recurring question related to the ubiquitin system has been whether substrates other than ubiquitin may be used in parallel pathways to regulate as yet unrecognized cellular activities.

Here, we report the identification of a novel 90-kD form of the Ran-GTPase–activating protein RanGAP1. By peptide sequence analysis and using specific mAbs, the 90-kD RanGAP1 was identified as a modified form of the previously described 70-kD RanGAP1. The modification was found to consist of a covalent ligation between RanGAP1 and a novel ubiquitin-like protein, and it could be reversed by an enzymatic activity that cofractionated with the NPC. Moreover, the 90-kD modified form of RanGAP1 was itself localized to the NPC, and more specifically, to the cytoplasmic fibers of the NPC. In contrast, the 70-kD unmodified form of RanGAP1 was strictly cytoplasmic. Thus, a novel ubiquitin-like modification appears to modulate the partitioning of RanGAP1 between the cytosol and the NPC.

Materials and Methods

Isolation and Fractionation of Rat Liver Nuclear Envelopes

Rat liver nuclei were isolated as described (Blobel and Potter, 1966) and stored frozen at −80°C in 100-U aliquots (1 U = 3 × 10^6 nuclei). Nuclear envelopes were prepared by a modification of the procedure described by Dwyer and Blobel (1976). Nuclei were thawed and pelleted at 500 rpm in a tabletop microfuge for 1 min. After removing the supernatant, the pellet was resuspended to a final concentration of 100 U/ml by dropwise addition of cold buffer A (0.1 mM $MgCl_2$, protease inhibitors [0.5 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 18 μg/ml aprotinin], 5 μg/ml DNase I (Sigma Chemical Co., St. Louis, MO), and 5 μg/ml RNase A [Sigma Chemical Co., St. Louis, MO]) with constant vortexing. The nuclei were then immediately diluted to 20 U/ml by the addition of ice-cold buffer B (10% sucrose, 20 mM triethanolamine, pH 8.5, 0.1 mM $MgCl_2$, 1 mM DTT, and protease inhibitors), again with constant vortexing. The suspension was dounced four times in a glass dounce homogenizer (tight pestle) and incubated at room temperature for 15 min. After the 15-min incubation, the suspension was underlaid with 5 ml of ice-cold buffer C (30% sucrose, 20 mM triethanolamine, pH 7.5, 0.1 mM $MgCl_2$, 1 mM DTT, and protease inhibitors) and centrifuged at 2,600 g in a swinging bucket rotor (model type HB-4; Sorvall, Wilmington, DE) for 15 min at 4°C. After removing the supernatant and sucrose cushion, the pellet was resuspended to a final concentration of 100 U/ml in ice-cold buffer D (10% sucrose, 20 mM triethanolamine, pH 7.5, 0.1 mM $MgCl_2$, 1 mM DTT, and protease inhibitors). The suspension was dounced as described above, and diluted to 66 U/ml by the addition of cold buffer C plus 0.3 μg/ml heparin (Sigma Chemical Co.). The suspension was immediately underlaid with 5 ml of buffer C and pelleted as described above. The pellet resulting from this second extraction is operationally defined as the nuclear envelope fraction. The pore complex lamina fraction (PCLF) was derived from the nuclear envelope fraction by extraction with 1% Triton X-100 and 0.025% SDS, followed by an additional round of centrifugation as described above.

Conditions for Empigen BB extraction of the PCLF were as follows: The PCLF was resuspended in cold buffer D (minus DTT) at 100 U/ml and divided into two samples, one for mock *N*-ethylmaleimide (NEM) treatment and one for NEM treatment. To the mock NEM–treated sample, DTT was added to a final concentration of 10 mM, and NEM (pre-

pared as a 1 M stock in dimethyl sulfoxide immediately before use) was subsequently added to a final concentration of 5 mM. To the NEM-treated sample, NEM alone was added to 5 mM. Both samples were incubated on ice for 5 min and then extracted by dilution of the samples to 66 U/ml with buffer D containing 0.9% Empigen BB (Calbiochem/Novabiochem Corp., La Jolla, CA). Samples were incubated on ice for 5 min and subsequently pelleted for 15 min at 15,000 rpm. After precipitation with 10% TCA, the samples were resuspended in sample buffer, and the equivalent of 10 U of nuclei were analyzed by immunoblot analysis.

Preparation of mAbs

mAbs 19C7, 19G12, and 2F1 were prepared by immunizing BALB/c mice with recombinant protein (prepared as described below) consisting of the first 203 amino-terminal residues of mouse RanGAP1. mAb 21C7 was produced by immunizing mice with the full-length recombinant GMP1, prepared as described below. Hybridoma production and screening and ascites production were performed as described previously (Choi and Dreyfuss, 1984). Antibody specificities were determined by immunofluorescence and immunoblot analysis.

Gel Electrophoresis and Immunoblot Analysis

Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane as described previously (Dreyfuss et al., 1984). Membranes were blocked in 5% nonfat dry milk in PBST (137 mM $NaCl_2$, 2.7 mM KCl_2, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 0.1% Tween 20). mAb ascites fluids were diluted 1:2,000 in PBST containing 2% BSA. Antibodies were detected using luminol-based chemiluminescence.

Peptide Sequence Analysis

Rat liver nuclear envelopes were separated by SDS-PAGE (10% acrylamide) and transferred to a polyvinylidene difluoride membrane. The 90-kD Ran-GTP–binding protein was identified by staining with Ponceau S, cut from the membrane, and digested with endoproteinase Lys-C. Peptides were separated and sequenced as described (Fernandez et al., 1992).

Immunofluorescence Microscopy

Buffalo rat liver (BRL) cells grown on coverslips were washed in PBS and either fixed in 2% formaldehyde/PBS for 30 min at room temperature and permeabilized with −20°C acetone for 3 min, or were permeabilized with 50 μg/ml digitonin (Aldrich Chemical Co., Milwaukee, WI) in transport buffer (20 mM Hepes-KOH, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 2 mM DTT, and protease inhibitors) for 5 min at room temperature, followed by fixation in 2% formaldehyde/PBS for 30 min at room temperature. Primary antibodies were diluted 1:2,000 in 2% BSA/PBS and incubated with the fixed cells for 1 h at room temperature. After washing with PBS, the cells were incubated with fluorescein-conjugated goat anti–mouse (Organon/Teknika, Durham, NC) for 30 min at room temperature, washed again in PBS, and mounted in buffer containing 80% glycerol, 50 mM Tris-HCl, pH 8.0, and 0.1% *p*-phenylenediamine.

Immunopurifications

100 U of rat liver nuclear envelopes in buffer D (200 U/ml) were solubilized by addition of SDS to a final concentration of 0.5% and by heating at 95°C for 10 min. 4 vol of RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate) were subsequently added to the sample followed by centrifugation at 20,000 *g* for 20 min. The supernatant was split into two tubes and incubated for 1 h at 4°C with 1 μl of mAb 21C7 ascites fluid or with 1 μl of ascites fluid produced by mice injected with the parent myeloma cell line SP2/0. 20 μl of protein G–Sepharose (Pharmacia Biotech Inc., Piscataway, NJ) was then added and the samples were incubated for an additional hour at 4°C. After brief centrifugations, the resins were washed four times with ice-cold RIPA buffer/0.1% SDS, once time with PBS, and then boiled in SDS sample buffer. The equivalent of 20 U of nuclei were used for immunoblot analysis.

Bacterial Expression of Recombinant Proteins

DNA fragments encoding amino acids 1–203 of mouse RanGAP1 or the entire open reading frame of expressed sequence tagged (EST) clone #220 (starting with the first in frame methionine) were generated by PCR and subcloned into the NdeI and AvaI sites of the bacterial expression vector pET21a (Novagen, Inc., Madison, WI). The expression vectors were transfected into the *Escherichia coli* strain BL21 (DE3) pLysE, and recombinant proteins were induced for 3 h at 37°C by the addition of 0.2 mM isopropyl thio-β-D-galactoside (IPTG) to the culture media. Bacteria were harvested, lysed in 6 M guanidine hydrochloride, and recombinant proteins were purified by Ni-NTA agarose as recommended by the manufacturer (QIAGEN Inc., Chatsworth, CA). Purified proteins were TCA precipitated and resuspended in PBS.

Immunogold EM

Rat liver nuclear envelopes (prepared as described above) were processed for immunogold EM as described (Wu et al., 1995). Envelopes were incubated with a mixture of mAbs 19C7, 19G12, and 2F1, each diluted 1:1,000.

Results

Identification of a 90-kD Form of RanGAP1 Associated with Isolated Nuclear Envelopes

To evaluate the role of Ran in translocation of substrates through the NPC in more detail, we have characterized proteins that are associated with the NPC and that interact directly with Ran-GTP. Two elements were used in identifying proteins with these credentials: (*a*) a subcellular frac-

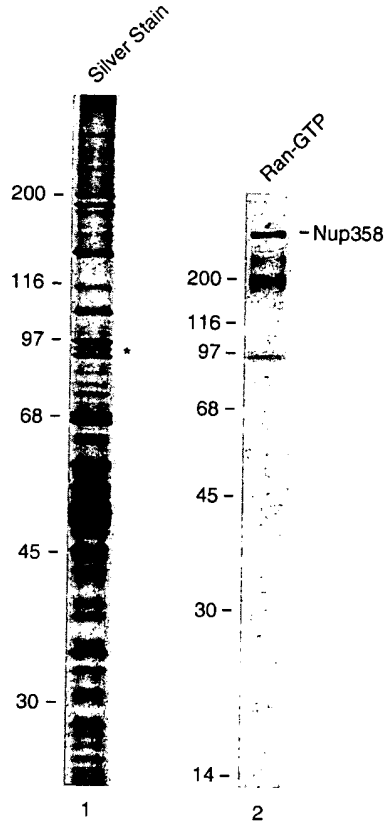


Figure 1. Identification of a 90-kD Ran-GTP–binding protein associated with isolated nuclear envelopes. Rat liver nuclear envelopes were isolated and separated by SDS-PAGE (10% acrylamide). Proteins were visualized by silver stain (lane *1*), or were transferred to nitrocellulose membrane and probed with [^{32}P]GTP-labeled Ran (lane *2*), as described (Coutavas et al., 1993). The asterisk in lane *1* indicates the position of the 90-kD protein identified in lane *2*, and the protein subjected to peptide sequence analysis.

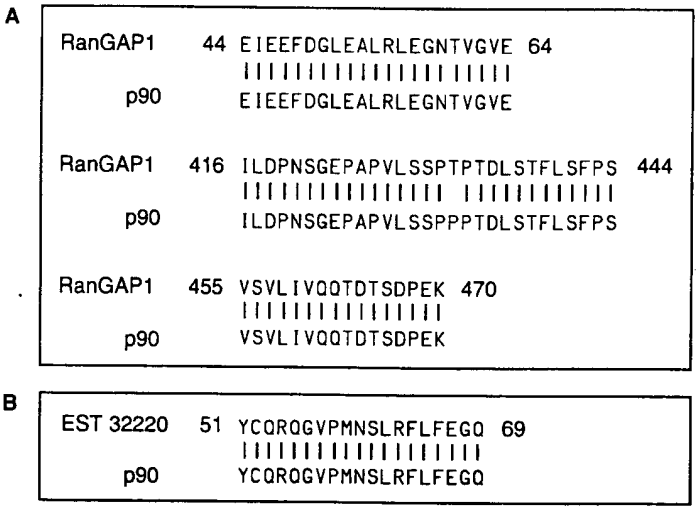
tion highly enriched in nucleoporins; and (*b*) a ligand blot assay using radiolabeled Ran-GTP. Starting with purified rat liver nuclei, a highly enriched nuclear envelope fraction that contained all of the currently known nucleoporins and that was essentially free of contaminating chromatin and chromatin-associated proteins was isolated (Fig. 1, lane *1*). When proteins in this fraction were transferred to a nitrocellulose membrane and probed with radiolabeled Ran-GTP, four prominent bands were detected, corresponding to proteins with apparent molecular masses of 350, 250, 180, and 90 kD (Fig. 1, lane *2*). The highest molecular mass protein corresponds to the previously characterized nucleoporin, Nup358 (Wu et al., 1995; Yokoyama et al., 1995), while the proteins of 250 and 180 kD remain uncharacterized. All of the identified proteins bound specifically to Ran-GTP, since their binding was competed with excess cold Ran-GTP, but not with excess cold Ran-GDP (data not shown). Using this same assay, Lounsbury et al. (1994) have identified a similar subset of nuclear Ran-GTP–binding proteins.

The nuclear envelope–associated Ran-GTP–binding protein migrating at 90 kD was recognized as a single protein (proteins were stained with Ponceau S and their positions were marked before incubation with Ran-GTP), and it was subjected to peptide sequence analysis. Three peptides, encompassing a total of 66 amino acids, were virtually identical to a protein that had been characterized previously as a Ran GTPase–activating protein and known as Fug1 (DeGregori et al., 1994) or RanGAP1 (Bischoff et al., 1995*a*) (Fig. 2 *A*). The peptides were derived from regions spanning nearly the entire 589 amino acids predicted for RanGAP1, ranging from amino acids 44 to 470. In addition to the three peptides identical to RanGAP1, a fourth peptide sequence was obtained that showed no homology to the predicted amino acid sequence of RanGAP1, but that was encoded by a human EST cDNA, clone 32220 (Fig. 2 *B*).

cDNAs predicting a 65-kD RanGAP1 have been isolated from both human and mouse sources (DeGregori et al., 1994; Bischoff et al., 1995). Furthermore, human RanGAP1 purified from HeLa cells migrates (after eight fractionation procedures) as a 65-kD polypeptide by SDS-PAGE (Bischoff et al., 1994). To demonstrate further that the protein we identified was homologous to RanGAP1, mAbs were generated against a recombinant protein corresponding to the first 203 amino acids encoded by the mouse RanGAP1 cDNA. Hybridomas producing antibodies specific for RanGAP1 were selected by immunofluorescence and immunoblot analysis. All of the identified mAbs recognized a 90-kD protein (which on high resolution gels appeared as three closely spaced proteins) in the nuclear envelope fraction that comigrated with the protein detected with labeled Ran-GTP (Fig. 2 *C*). Our identification of a 90-kD protein homologous to RanGAP1, as well as previous work identifying a 65-kD protein (Bischoff et al., 1994), suggest the existence of two highly related RanGAP1 molecules. The identification of one peptide with no homology to RanGAP1 suggested three possible origins for the 90-kD protein. These include a unique gene-encoding protein highly related to RanGAP1, an alternatively spliced message derived from the RanGAP1 gene, or a posttranslational modification involving the attachment of an independently encoded polypeptide to RanGAP1.

Detection of Two Forms of RanGAP1 and Their Localization to Distinct Subcellular Domains

Because the nuclear envelope–associated RanGAP1 dif-



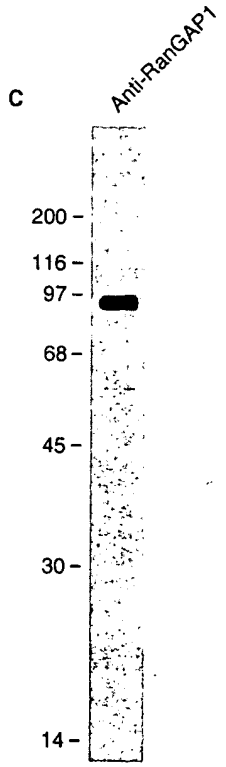


Figure 2. The 90 kD RanGTP–binding protein associated with isolated nuclear envelopes is homologous to RanGAP1. (*A*) Alignment of peptide sequences derived from the 90-kD Ran-GTP-binding protein (p90) with predicted amino acid sequences of mouse RanGAP1. (*B*) Alignment of unique peptide sequence derived from p90 with the predicted amino acid sequence derived from EST clone 32220. (*C*) Immunoblot analysis of isolated nuclear envelope proteins with the RanGAP1-specific mAb 19C7.

fered in size from the previously characterized protein, we used the mAbs against RanGAP1 to characterize it in cultured cells. Immunofluorescence microscopy on formaldehyde-fixed and acetone-permeabilized BRL cells revealed a diffuse cytoplasmic signal, as well as an intense nuclear rim staining, further demonstrating an association of RanGAP1 with the nuclear envelope (Fig. 3 *a*). When cells were treated with digitonin (a detergent that specifically permeabilizes the plasma membrane, but not the nuclear envelope) before fixation, and then stained with antibodies, the cytoplasmic signal was no longer apparent, indicating an extraction of the cytoplasmic RanGAP1 (Fig. 3 *b*). Nuclear rim staining, however, was still evident and its punctate appearance was very similar to that observed with antibodies specific for nucleoporins (Davis and Blobel, 1986). Because the nuclear envelope is not permeabilized by digitonin, detection of RanGAP1 under these conditions suggests an association with the cytoplasmic side of the nuclear envelope. During mitosis, RanGAP1 was detected throughout the cell, but surprisingly, it was also found associated with the mitotic spindles. Although association with the spindles could be detected in cells fixed and then permeabilized (Fig. 3 *a*), the localization was most obvious when cells were permeabilized with digitonin before fixation (Fig. 3, *c–f*). By early telophase, RanGAP1 was detected as a halo around the surface of the newly condensed chromatin (Fig. 3 *f*).

Antibodies against RanGAP1 were next used for immunoblot analysis. When total BRL cell lysate was probed with antibodies against RanGAP1, two proteins with apparent molecular masses of 90 and 70 kD were detected (Fig. 4, lane *1*), confirming the presence of two related forms of RanGAP1. Similar to the multiple bands detected in isolated nuclear envelopes, the 90-kD protein was actually three closely spaced proteins, whereas the 70-kD protein resolved into at least two proteins. As demonstrated above, the cytoplasmic pool of RanGAP1 is extracted with digitonin, leaving RanGAP1 association with the nuclear envelope intact. Immunoblot analysis of the digitonin-soluble and -insoluble fractions revealed a clear fractionation of the two forms of RanGAP1. Consistent with data presented above on isolated nuclear envelopes, only the 90-kD form of RanGAP1 was detected in the digitonin-insoluble fraction (Fig. 4, lane *2*). Conversely, the 70-kD form of RanGAP1 was the predominant form detected in the soluble fraction (Fig. 4, lane *3*) along with a relatively minor amount of the 90-kD form (15% of the total). Together, the immunoblot and immunofluorescence data demonstrate that the 70-kD form of RanGAP1 is completely cytoplasmic, and that the 90-kD form of RanGAP1 is associated predominantly with the nuclear envelope.

To localize further the nuclear envelope–associated 90-kD form of RanGAP1, immunogold EM was per-

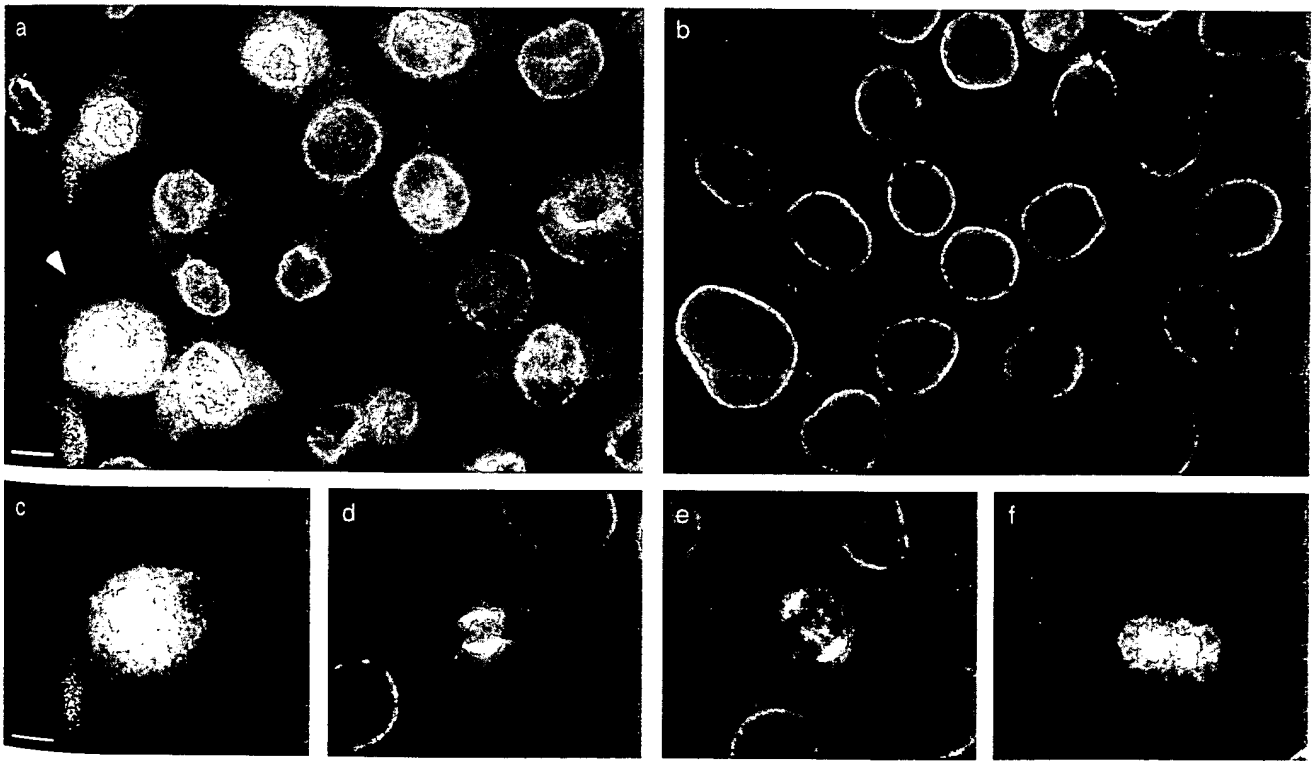


Figure 3. RanGAP1 localizes to the cytoplasm and to the nuclear envelope in interphase cells, and during mitosis, it associates with the mitotic spindles. BRL cells were grown on coverslips, fixed, and permeabilized as indicated before incubation with mAb 19C7. (*a*) Interphase cells first fixed with formaldehyde and then permeabilized with acetone. The arrow indicates a metaphase cell in which staining of the mitotic spindles is visible. (*b*) Interphase cells first permeabilized with digitonin followed by formaldehyde fixation. (*c*) A cell in prophase, first fixed with formaldehyde and then permeabilized with acetone. (*d*) A metaphase cell first permeabilized with digitonin and then fixed with formaldehyde. (*e*) An anaphase cell first permeabilized with digitonin and then fixed with formaldehyde. (*f*) A cell in telophase, first permeabilized with digitonin and then fixed with formaldehyde. Bars: (*a* and *b*) 30 μm; (*c–f*) 8 μm.

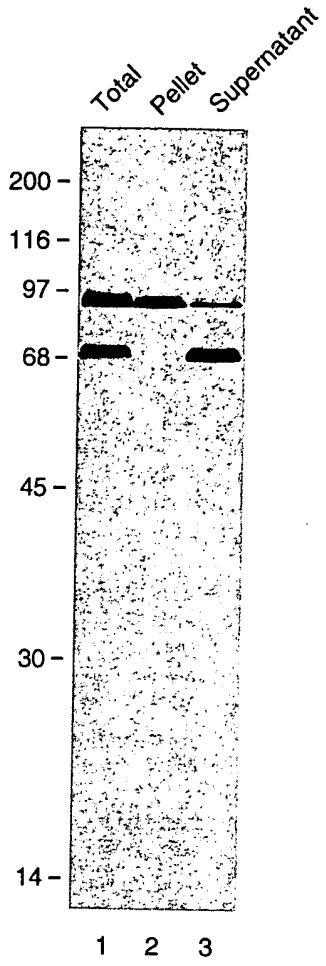


Figure 4. Two forms of RanGAP1 are detected, and they fractionate with distinct subcellular domains. BRL cells were either lysed directly in SDS-PAGE sample buffer (lane *1*), or they were fractionated into a digitonin-insoluble fraction (lane *2*) and a digitonin-soluble fraction (lane *3*). Proteins were separated by SDS-PAGE (12.5% acrylamide), transferred to nitrocellulose membrane, and probed with mAb 19C7.

formed using isolated nuclear envelopes and RanGAP1-specific antibodies (Fig. 5). The 90-kD form of RanGAP1 localized to the NPCs (consistent with biochemical subfractionation; data not shown), with 95% (total = 1547) of the signal detected in association with cytoplasmic aspects of the NPC. Similar to the localization reported previously for Nup358 (Wu et al., 1995; Yokoyama et al., 1995), the 90-kD form of RanGAP1 appeared to localize at or near the tips of the cytoplasmic fibers of the NPC (Fig. 5 *C*). In addition to this predominant localization, 5% of the total signal was associated with the nucleoplasmic face of the NPC. The low level of signal was not a result of inaccessibility of the antibodies to the nucleoplasmic side of the nuclear envelopes, as determined by labeling with antibodies to both Nup153 and the nuclear lamins (data not shown). The 90-kD form of RanGAP1, therefore, localizes definitively to the cytoplasmic fibers of the NPC and possibly to the nucleoplasmic side of the NPC. In summary, two forms of RanGAP1 have been identified: a 70-kD form localizing specifically to the cytoplasm and corresponding to the previously characterized 65-kD RanGAP1, and a 90-kD form localizing predominantly to the cytoplasmic fibers of the NPC.

Identification of a Novel Ubiquitin-like Polypeptide Associated with RanGAP1

To further investigate the relationship between the 90-kD form of RanGAP1 and the previously characterized 65-kD form of RanGAP1, we focused on EST clone 32220, which coded for the one novel peptide sequence described above. The 1.5-kb cDNA clone was obtained from the IMAGE consortium (Lawrence Livermore National Laboratory, Livermore, CA), and an open reading frame was identified starting at the 5′ end and extending through nucleotide 386 (Fig. 6 *A*). A putative initiation codon begins with nucleotide 100, and surrounding nucleotides conform to the consensus for translation initiation (Kozak, 1991). Beginning with this methionine, the open reading frame codes for a protein with a predicted molecular mass of 11.5 kD. When expressed in bacteria, the recombinant protein migrated with an apparent molecular mass of 17 kD by SDS-PAGE, similar to the presumptive free form of the protein identified in rat liver nuclei (see Fig. 10; data not shown). The protein encoded by this open reading frame will be referred to as GMP1 (GAP modifying protein 1). A search of sequence data bases for proteins homologous to GMP1 revealed a family of highly related proteins in a wide range of organisms, including plants (*Oryza sativa*, *Arabitopsis thaliana*, and *Brassica campestris*), worms (*Caenorhabditis elegans*), protozoans (*Plasmodium falciparum* and *Toxoplasma gondii*), and fungi (*S. cerevisiae*). While the majority of sequences were incomplete translations predicted from EST clones, full-length open reading frames were derived from several overlapping clones (Fig. 6 *B*). Based on the sequence similarities between homologues, GMP1 could be divided into three domains. The amino-terminal domain (defined by the first 20 amino acids) varies greatly between homologues, while the second domain, extending from amino acid 20 to a carboxyl-terminal double glycine, is highly conserved. All of the proteins contained a third domain of variable sequence and length, extending beyond the carboxyl-terminal double glycine. Among mammals, human, mouse, and rat GMP1 were found to be 100% identical, and human cDNAs encoding two additional GMP1-related proteins were also identified. A full-length open reading frame was deduced from one of these clones, and it predicts a protein that is 45% identical to GMP1 throughout its entire length and 53% identical throughout its carboxyl-terminal domain (Mannen et al., 1996). Overall identities between GMP1 and the worm, rice, and yeast homologues are 59, 39, and 40%, respectively, whereas identities between the carboxyl-terminal domains are 64, 42, and 46%, respectively. Interestingly, the yeast homologue (Smt3p) was originally identified as a suppressor of *MIF2*, a gene encoding a centromere-associated protein required for mitotic spindle integrity (Meluh and Koshland, 1995).

The homology of most significance, however, was that between GMP1 and ubiquitin (Fig. 6 *B*). The homology with ubiquitin began after the divergent amino-terminal domain found in GMP1 (which is absent from ubiquitin) and extended over the entire length of the second domain. Furthermore, a signature feature of ubiquitin, an invariable double glycine at the carboxyl terminus, is conserved in GMP1 and in all of the GMP1-related proteins (Fig. 6 *B*). This double glycine is essential for the proteolytic processing of ubiquitin precursors and for the recycling of post-translationally synthesized polyubiquitin (Hershko and Ciechanover, 1992; Wilkinson, 1995). Similar to ubiquitin

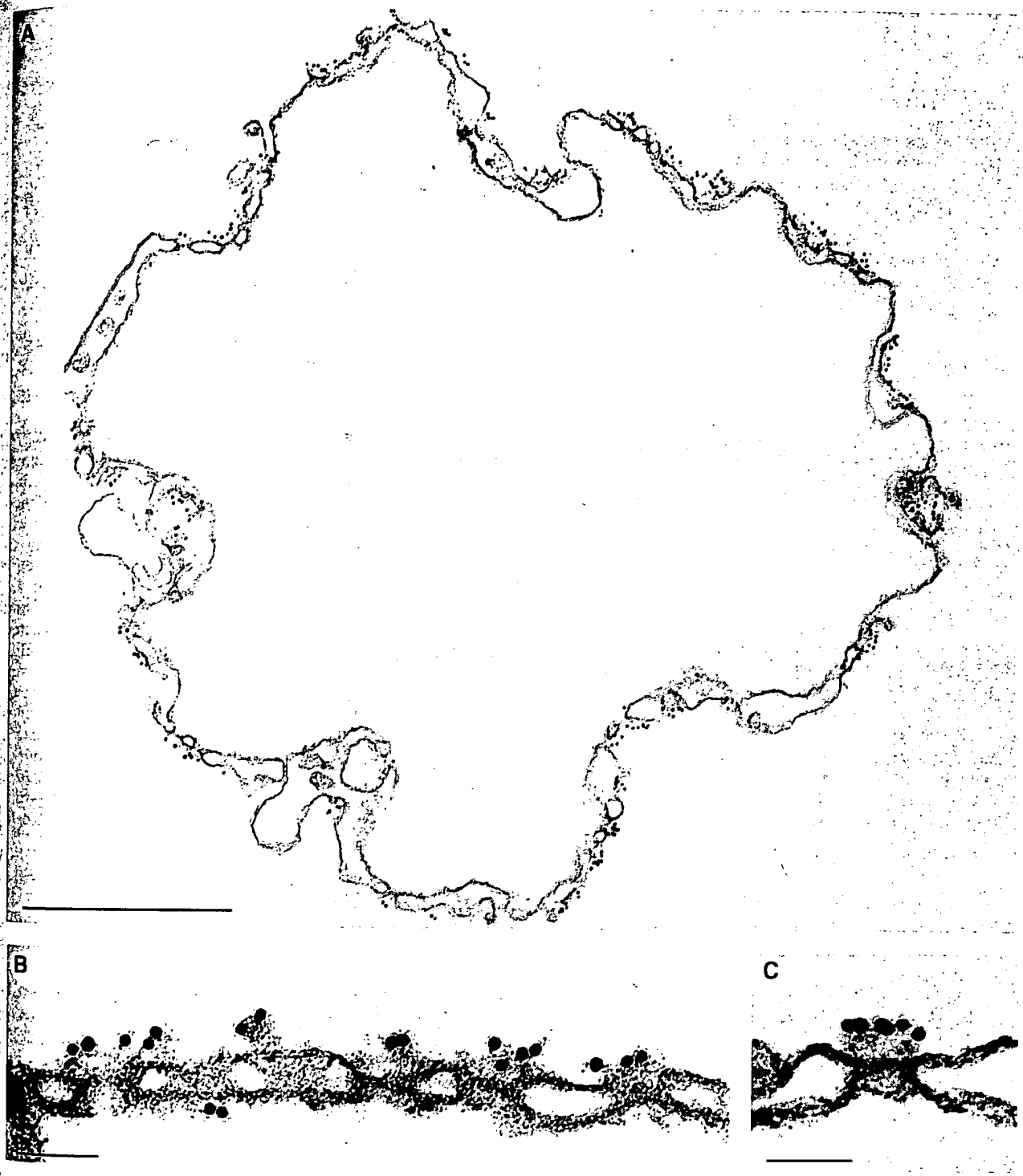


Figure 5. The 90-kD form of RanGAP1 localizes to the cytoplasmic fibers of the NPC. Isolated nuclear envelopes were fixed and incubated with a combination of three RanGAP1-specific mAbs (19C7, 2F1, and 19G12) followed by incubation with goat anti-mouse IgG conjugated to 10-nm gold. Samples were processed for thin sectioning and observed by EM. (*A*) A section of nuclear envelope demonstrating the typical labeling pattern observed. The cytoplasmic side of the nuclear envelope (evidenced by occasional blebs in the membrane) is oriented towards the outside. (*B*) A high magnification view along a single length of envelope. The cytoplasmic side of the envelope is oriented towards the top. (*C*) A high magnification view of a single NPC, demonstrating localization to the tips of the cytoplasmic fibers. Bars: (*A*) 1 μm; (*B* and *C*) 0.1 μm.

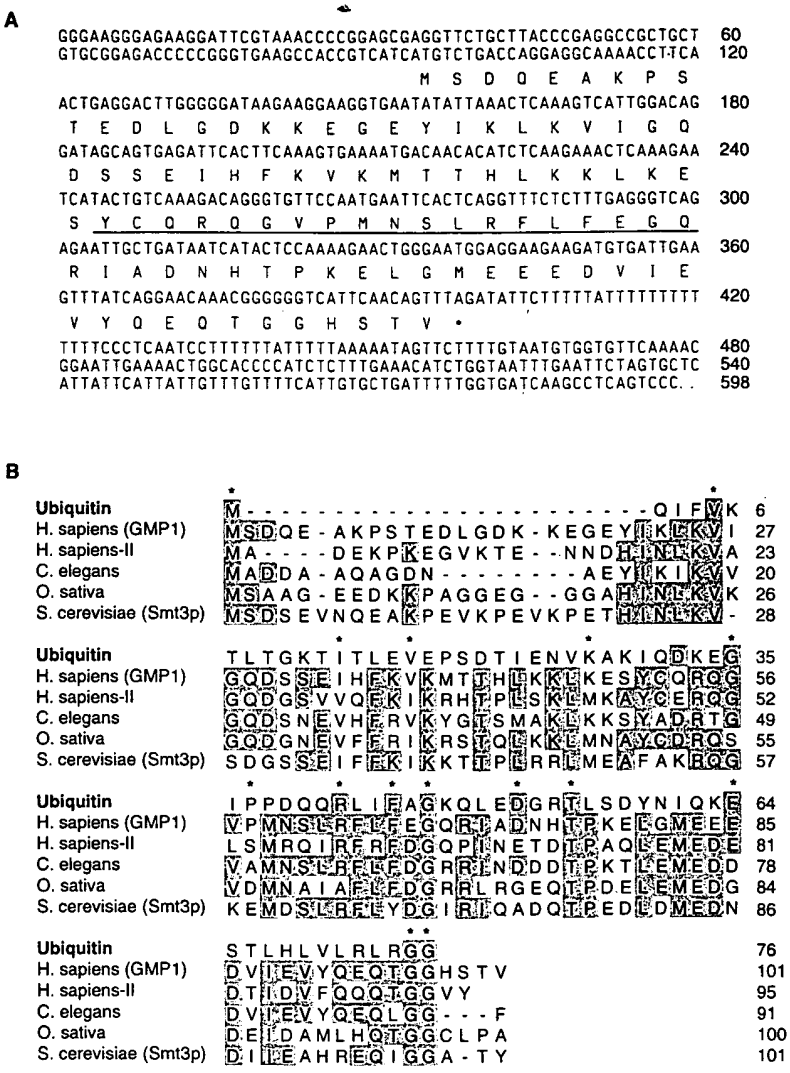


Figure 6. GMP1 is homologous to ubiqui[tin] and it is highly conserved from human to ye[ast]. (*A*) Partial DNA sequence of EST cDNA clone 32220 and the predicted amino acid sequence of GMP1. The underlined amino acids were also derived from peptide sequence analysis (see Fig. 2 *B*). (*B*) Amino acid sequence alignments between human ubiquitin, GMP1, and GMP1-related proteins. Residues identical in three or more of the aligned proteins are shaded. Asterisks indicate identical residues in GMP1 and ubiquitin. The primary sequence of human ubiquitin is as first described by Schlesinger et al. (1975). The remaining sequences, with the exception of *H. sapiens II* and the yeast homologue, were identified as predicted translations of EST clones. The human GMP1-related protein (*H. sapiens II*) was derived from the HSMT3 cDNA (Mannen et al., 1996). The *C. elegans* homologue was compiled from clones with GenBank accession Nos. D76147, D73158, and T02042, and the rice homologue (*O. sativa*) was derived from clones D15376 and D22620. The yeast sequence (*S. cerevisiae*) is derived from a cDNA clone coding Smt3p (Meluh and Koshland, 1995).

precursors (all known ubiquitins are synthesized as precursors with carboxyl-terminal extensions, or as polyubiquitin), the double glycines present in GMP1 and in the GMP1-related proteins are not at the extreme carboxyl terminus. The presence of this double glycine in the carboxyl-terminal domain of GMP1, followed by a short variable domain, suggests that GMP1 and ubiquitin may undergo similar enzymatic transformations. At the same time, the overall identity with ubiquitin is only 18%, identifying GMP1 as a novel ubiquitin-like protein.

To further characterize GMP1, recombinant protein was produced in bacteria and used to generate mAbs. GMP1-specific antibodies were identified by immunofluorescence and immunoblot analysis. The antibodies were first used to demonstrate that GMP1 and the 90-kD form of RanGAP1 are covalently associated. Nuclear envelope–associated RanGAP1 was immunopurified after denaturation in SDS with a RanGAP1-specific mAb. Immunoblot analysis of the immunopurified protein was then performed using an mAb against GMP1 (the specificity of these antibodies is demonstrated in Fig. 10). Antibodies against GMP1 recognized a 90-kD protein among total nuclear envelope proteins (Fig. 7, lane *1*) and a 90-kD polypeptide immunopurified with the anti-RanGAP1 antibody (Fig. 7, lane *2*). No signal was detected among proteins immunopurified with the control antibody SP2/0 (Fig. 7, lane *3*). These results indicate that RanGAP1- and GMP1-specific antibodies recognize the same protein or two covalently associated proteins. The homology between GMP1 and ubiquitin is suggestive of the latter possibility. Furthermore, Northern blot analysis with probes specific for GMP1 and RanGAP1 revealed distinct mRNA transcripts of 1.4 and 3.0 kb, respectively. No indication of a single, common transcript encoding both proteins was detected by Northern blot or reverse-transcription PCR (data not shown).

GMP1 Colocalizes to the NPC with RanGAP1 and It Is Also Present in the Nucleus

Immunofluorescence microscopy on formaldehyde-fixed and acetone-permeabilized BRL cells detected GMP1 in the nucleus and associated with the nuclear envelope (Fig. 8 *a*). The intranuclear signal was largely homogeneous; however, the antigen was also concentrated in foci that varied widely in number and in intensity from cell to cell. The signal associated with the nuclear envelope was most apparent when the cells were permeabilized with digitonin before fixation and labeling, and it was very similar to the

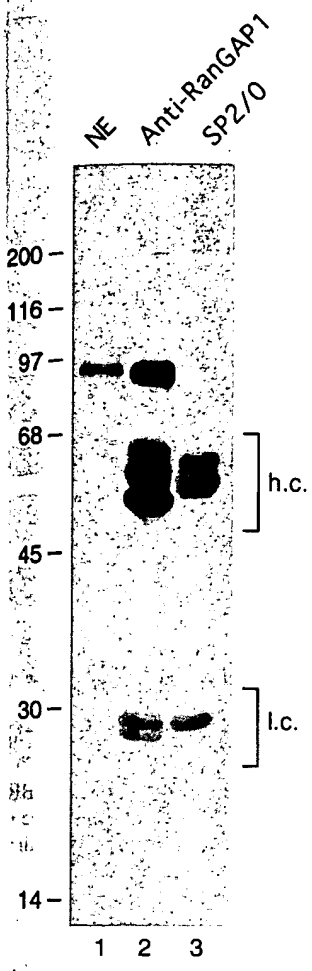


Figure 7. RanGAP1 and GMP1 are covalently associated. Isolated nuclear envelopes were solubilized with SDS (lane *1*), and the 90-kD form of RanGAP1 was immunopurified with the RanGAP1-specific mAb 19C7 (lane *2*). A control immunopurification was performed with antibody derived from the myeloma cell line SP2/0 (lane *3*). Proteins were separated by SDS-PAGE (12.5% acrylamide), transferred to nitrocellulose membrane, and probed with the GMP1-specific mAb 21C7. Antibody heavy chains (*h.c.*) and light chains (*l.c.*) are indicated on the right.

ignal observed with antibodies to RanGAP1 (Figs. 3 *b* nd 8 *b*). Also, during mitosis, the localization of GMP1 ppeared very similar to that of RanGAP1. Signal was detected throughout the cells concomitant with the breakdown of the nuclear envelope at prophase (Fig. 8 *c*), but lso associated with the mitotic spindles during metaphase nd anaphase (Fig. 8, *d* and *e*). By early telophase, GMP1 ould be seen concentrated around the newly condensed hromatin (Fig. 8 *f*). Like the 90-kD form of RanGAP1, MP1 was also sublocalized to the NPC by immunogold M (data not shown).

Immunoblots of total BRL cell lysate probed with antiodies against GMP1 revealed a major signal at 90 kD, dentifying the 90-kD form of RanGAP1 as the primary ellular protein associated with GMP1 (Fig. 9, lane *1*). Sigificantly less prominent proteins were also detected, however, including a polypeptide with an apparent molecular ass of 50 kD, and a large number of proteins migrating bove 116 kD. All of these proteins are presumably covant conjugates formed between GMP1 and other cellular roteins. To determine the subcellular localization of these roteins, digitonin-soluble and -insoluble fractions (similar o those in Fig. 4) were also probed. As expected for the 0-kD form of RanGAP1, the 90-kD protein recognized y GMP1-specific antibodies was also found predomiantly in the digitonin-insoluble fraction (Fig. 9, lane *2*). he high molecular mass proteins fractionated exclusively with the digitonin-insoluble fraction (Fig. 9, lane *2*), and these antigens likely account for the intranuclear signal observed by immunofluorescence microscopy. The protein migrating at 50 kD, on the other hand, fractionated exclusively with the digitonin-soluble fraction (Fig. 8, lane *3*) and is likely to be a cytoplasmic protein. Only a very weak signal was detected at 17 kD, presumably representing free GMP1. The levels of free GMP1 may be underrepresented, since it is possible that the antibody recognizes conjugated GMP1 better than it does free GMP1 (see Fig. 10). These data further support the association of GMP1 with RanGAP1, and they demonstrate the association of GMP1 with additional cellular proteins, predominantly in the nucleus.

The Association between RanGAP1 and GMP1 Can Be Enzymatically Reversed by an Activity that Fractionates with NPCs

During the course of fractionating nuclear envelopes, it was observed that the 90-kD form of RanGAP1 consistently disappeared upon extraction of the PCLF (an insoluble fraction derived from extraction of isolated nuclear envelopes with Triton X-100) with the ionic detergent Empigen BB. Whereas the majority of nucleoporins present in the PCLF were quantitatively solubilized with Empigen BB, the 90-kD form of RanGAP1 was not detected in either the supernatant or the pellet fractions (Fig. 10, lanes *1* and *2*; pellet fraction not shown). Concomitant with the disappearance of the 90-kD form of RanGAP1, however, a 70-kD protein not observed in previous fractions appeared in the Empigen supernatant (Fig. 10, lanes *1* and *2*). Immunoblot analysis of the PCLF and the Empigen supernatant fractions with an antibody against RanGAP1 demonstrated that the 90-kD form of RanGAP1 had been precisely converted to a protein that comigrates with the 70-kD form (Fig. 10, lanes *3* and *4*). At the same time, the antigen recognized by GMP1-specific antibodies was converted to a protein with an apparent mass of 17 kD (Fig. 10, lanes *6* and *7*). Given the homologies between GMP1 and ubiquitin, this result can be interpreted as a "deubiquitination" of RanGAP1 by a specific protease that copurifies with the PCLF. To support this interpretation, the PCLF was preincubated with the sulfhydryl alkylating agent NEM before extraction with Empigen BB. NEM is a potent inhibitor of the peptidases responsible for cleaving ubiquitin/substrate isopeptide bonds (Johnson, E., personal communication). Consistent with the involvement of a specific peptidase in the release of GMP1 from RanGAP1, NEM greatly inhibited the conversions detected in mock-treated fractions (Fig. 10, lanes *5* and *8*). These data are consistent with the interpretation that the 90-kD form of RanGAP1 originates through a covalent and reversible linkage to GMP1 by a pathway that is analogous to ubiquitination.

Discussion

We have identified a novel ubiquitin-like modification that correlates with the partitioning of the Ran GTPase-activating protein RanGAP1 from the cytosol to the NPC. These findings have specific implications for the function

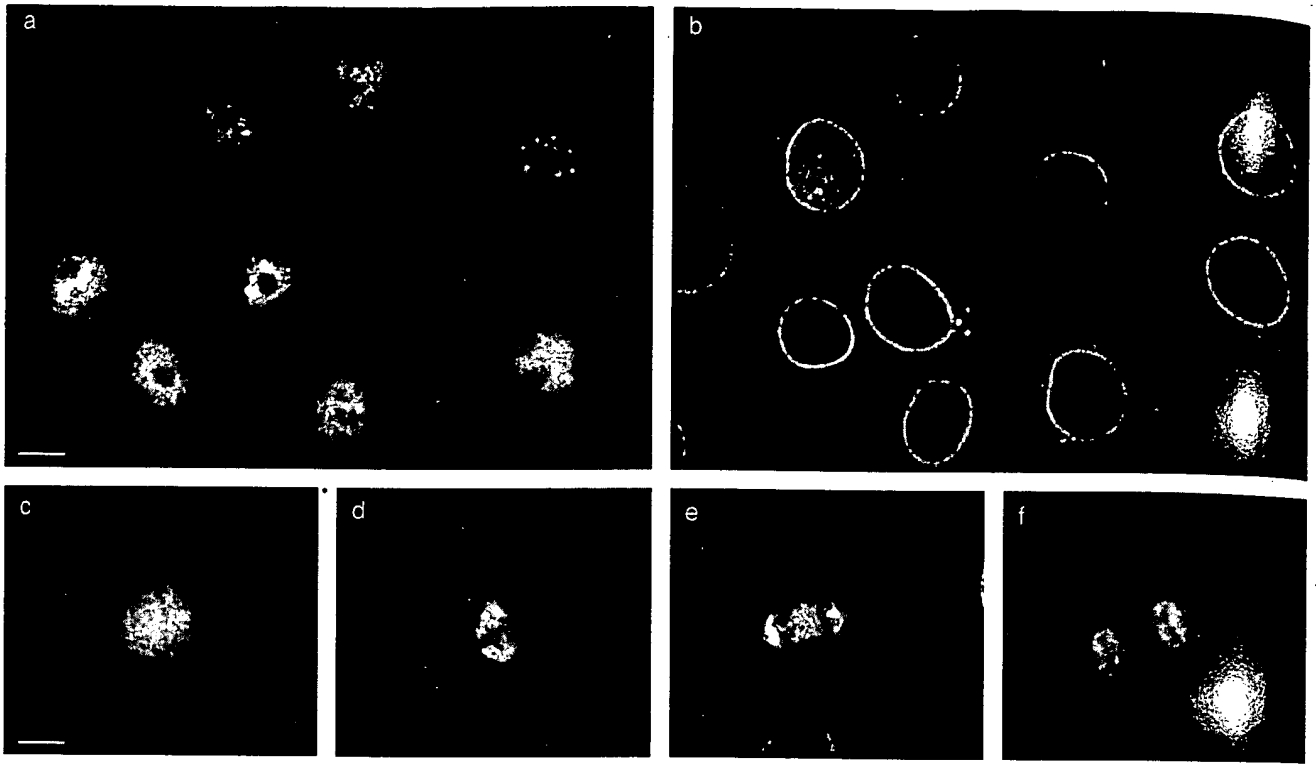


Figure 8. GMP1 localizes to the nucleus and the nuclear envelope during interphase, and to the mitotic spindle during mitosis. BRL cells were grown on coverslips, fixed, and permeabilized as indicated before incubation with mAb 21C7. (*a*) Immunofluorescence on cells fixed and permeabilized with acetone. (*b*) Localization of GMP1 in cells permeabilized with digitonin before fixation. (*c*) Localization in cells at prophase. Cells were first fixed and then permeabilized with acetone. (*d*) Localization in cells at metaphase. Cells were permeabilized with digitonin before fixation. (*e*) Localization at anaphase in cells permeabilized with digitonin before fixation. (*f*) Localization at telophase in cells permeabilized with digitonin before fixation. Bars: (*a* and *b*) 10 μm; (*c–f*) 8 μm.

of Ran in mediating nuclear import, and more general implications for the diversity of ubiquitin modifications and their functions. Ubiquitin is a 76–amino acid, highly conserved protein that is universally present in eukaryotic cells. Its covalent attachment to a variety of cellular proteins is catalyzed by a family of ubiquitin-conjugating (E2) enzymes that mediate the formation of an isopeptide bond between the carboxyl-terminal glycine of ubiquitin and the ϵ amino group of a lysine residue in an acceptor protein (Hershko and Ciechanover, 1992; Wilkinson, 1995). Equally important to their conjugation, ubiquitin subunits are also removed by ubiquitin carboxyl-terminal hydrolases (isopeptidases), making ubiquitination a reversible and regulatable posttranslational modification (Hershko and Ciechanover, 1992; Wilkinson, 1995). While ubiquitin-mediated proteolysis is the best-studied function of ubiquitin conjugation, it has also been implicated in regulating a host of other cellular processes, including endocytosis and vacuolar targeting, protein kinase activation, protein import into mitochondria, and peroxisome biogenesis (Ciechanover, 1994; Wilkinson, 1995; Chen et al., 1996; Egner and Kuchler, 1996; Galan et al., 1996; Hicke and Riezman, 1996; Roth and Davis, 1996; Strous et al., 1996). The utility of the ubiquitin system raises the question of whether parallel pathways exist, using novel ubiquitin-like proteins. At the present time, only several examples exist, including an IFN-inducible ubiquitin homologue that conjugates to a large number of intracellular proteins (Loeb and Haas, 1992), as well as a viral-encoded ubiquitin-like protein (Haas et al., 1996). Whereas the function of the IFN-inducible protein is currently uncertain, the viral-encoded ubiquitin-like protein may function to block degradation of short-lived proteins by the host.

Here, we report the existence of a new family of ubiquitin-like proteins that likely serve novel functions through their conjugation to specific protein substrates. We identified the first member of this family as a covalent modification of the 70-kD Ran GTPase–activating protein (RanGAP1, and we have named it GMP1 (for GAP modifying protein 1). Evidence for a covalent and reversible association between RanGAP1 and GMP1 included (*a*) derivation of peptide sequences for both RanGAP1 and GMP1 from a single protein migrating at 90 kD; (*b*) coimmunopurification of RanGAP1 and GMP1 antigens under protein-denaturing conditions; and (*c*) specific enzymatic conversion of the 90-kD form of RanGAP1 to the free 70-kD form of RanGAP1 and free GMP1. Evidence that the modification occurs posttranslationally included (*a*) detection of unique, nonoverlapping mRNAs coding for RanGAP1 and GMP1 by Northern blot analysis and RT-PCR; (*b*) overlapping but unique subcellular distributions of RanGAP1 and GMP1 in cultured cells; and (*c*) rapid, phosphorylation-dependent conversion of the 70-kD form of RanGAP1 to the 90-kD form in vivo (Matunis, M.J., and G. Blobel, unpublished results).

While the exact function of the ligation between RanGAP1

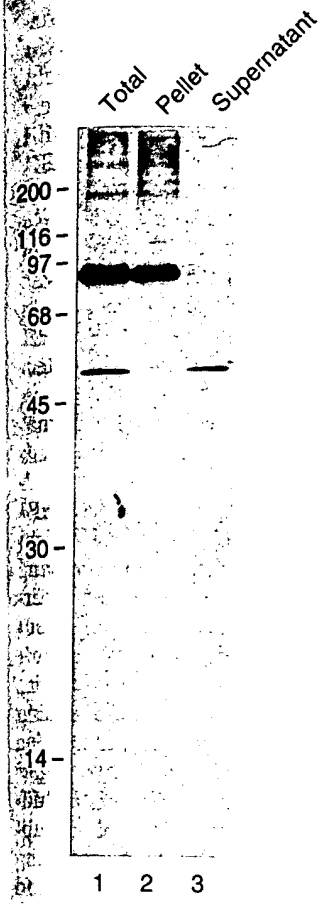


Figure 9. GMP1 associates predominantly with RanGAP1, but also with additional substrates in the nucleus and in the cytoplasm. BRL cells were either lysed directly in SDS-PAGE sample buffer (lane *1*), or were fractionated into a digitonin insoluble fraction (lane *2*), and a digitonin soluble fraction (lane *3*). Proteins were separated by SDS-PAGE (15% polyacrylamide), transferred to nitrocellulose membrane, and probed with mAb 21C7.

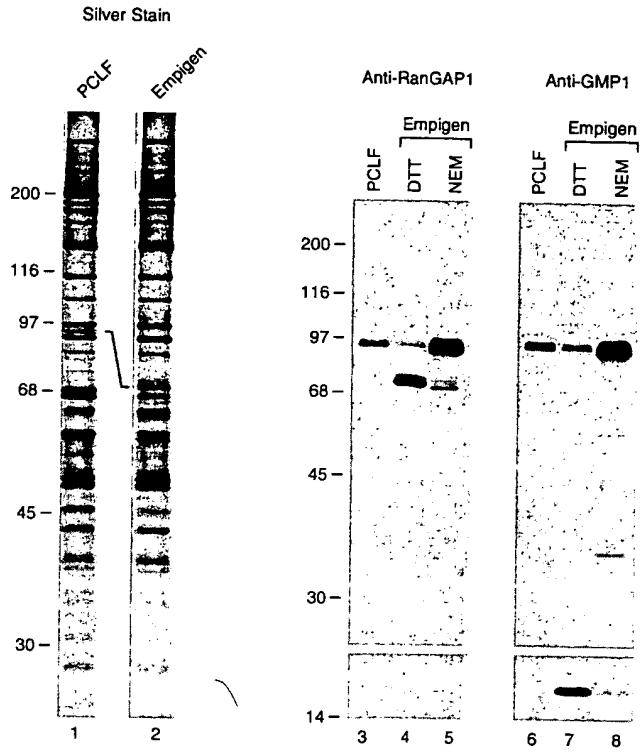


Figure 10. The 90-kD form of RanGAP1 is enzymatically processed into the 70-kD form of RanGAP1 and free GMP1. The pore complex lamina fraction (*PCLF*) was extracted with the ionic detergent Empigen BB after treatment with DTT and NEM, or after treatment with NEM alone. The PCLF and proteins solubilized by Empigen were separated by SDS-PAGE (10 or 12.5% acrylamide) and visualized by silver stain (lanes *1* and *2*), or they were transferred to nitrocellulose membrane and probed with the RanGAP1-specific mAb 19C7 (lanes *3–5*) or with the GMP1-specific mAb 21C7 (lanes *6–8*). Extraction with Empigen after mock treatment with NEM resulted in conversion of the 90-kD form of RanGAP1 to the 70-kD form (lanes *3* and *4*), with a concurrent shift in GMP1-reactive proteins from 90 to 17 kD (lanes *6* and *7*). Conversions were inhibited by preincubation of the PCLF with the alkylating agent NEM before Empigen extraction (lanes *5* and *8*). The lower portion of the immunoblots were overexposed, since the level of free GMP1 that was detected was not equivalent to the level of the 90-kD RanGAP1 (either resulting from a lower reactivity of the antibody for free GMP1 or from less free GMP1 being present on the blot).

and GMP1 remains to be determined, the modification correlated with localization of RanGAP1 to the NPC. Immunofluorescence and immunoblot analysis of digitonin-extracted cells revealed that while the modified form of RanGAP1 is predominantly associated with the nuclear envelope, the 70-kD unmodified form is strictly cytoplasmic. Approximately 15% of the 90-kD form of RanGAP1 was detected in the digitonin-extracted fraction; however, it remains to be determined whether this represents a true cytoplasmic pool, or more simply, protein released from the nuclear envelope during the fractionation procedure. Equivalent amounts of the 70-kD unmodified form of RanGAP1 and the 90-kD modified form of RanGAP1 were detected in whole lysates. Based on the apparent size of free recombinant GMP1 on SDS gels (17 kD), we estimate that a single copy of GMP1 is attached to RanGAP1. Unlike the majority of ubiquitin conjugates, no evidence for ligation of multiple copies of GMP1 to RanGAP1 was detected, and we have no evidence for a role in protein degradation. GMP1 may not form ubiquitin-like polymers because the lysine residues of ubiquitin that have been reported to form such chains (most commonly lysine 48) are not conserved in GMP1.

RanGAP1 has previously been purified from HeLa cells using a biochemical assay for RanGAP activity (Bischoff et al., 1994). Approximately equal levels of RanGAP activity were detected in nuclear and cytoplasmic fractions derived from interphase HeLa cells, and both activities were ultimately attributed to the 70-kD unmodified form of RanGAP1. Because our findings demonstrate that the unmodified form of RanGAP1 is strictly cytosolic, it is likely that the nuclear RanGAP1 purified in this study was converted from the GMP1-modified form to the unmodified form during the course of its purification (possibly during the 400 mM NaCl extraction of the nuclear pellet). Precedence for demodification of nuclear-associated RanGAP1 is provided by the specific release of GMP1 from RanGAP1 after detergent extraction of the isolated PCLF (Fig. 10). With this consideration, RanGAP activity detected in association with the nuclear pellet would indicate that the modified form of RanGAP1 is an active GAP. Because antibodies specific for mammalian RanGAP1 were not previously available, the 90-kD form of RanGAP1 was not detected by immunoblot analysis.

By immunogold EM, the 90-kD form of RanGAP1 was localized to the cytoplasmic fibers of the NPC. Approximately 5% of the signal detected on isolated nuclear envelopes was also localized to the nucleoplasmic side of the NPC. This relative distribution is consistent with results obtained by immunofluorescence microscopy, where the majority of the signal was detectable from the cytoplasmic side of the nuclear envelope. RanGAP1 has previously been considered a cytosolic factor, and models concerning how Ran functions to mediate nuclear import have taken this into account by postulating the existence of additional RanGAPs, or a requirement for Ran to shuttle between the nucleus and cytoplasm (Rush et al., 1996; Sazer, 1996). Localization of RanGAP1 to the cytoplasmic fibers of the NPC obviates the need for Ran to enter the cytoplasm to complete its GTP/GDP cycle, and strongly implicates the fibers as one of the major sites of RanGTPase activity and a critical regulatory point in protein import. We envision two models for Ran-mediated nuclear import based on Ran-GTP hydrolysis at this site. First, Ran-GTP hydrolysis at the cytoplasmic fibers of the NPC could be directly linked to release of docked karyopherin/substrate complexes and the commitment of these complexes to subsequent phases of translocation, as proposed recently (Melchior et al., 1995). Nup358, which is one component of the cytoplasmic fibers, potentially binds Ran-GTP and karyopherin/substrate complexes, and it would therefore be a likely site for this initial event. Subsequent steps in the translocation process would not require Ran, although Ran-GTP would have to be regenerated, presumably after translocation of Ran-GDP into the nucleus and interaction with RCC1. As an alternative model (although not necessarily exclusive), Ran-GTP hydrolysis could mediate the release and import of karyopherin/substrate complexes indirectly by simply serving to generate Ran-GDP. As recently demonstrated, the Ran-interactive protein p10 can function to coordinate dissociation of docked karyopherin receptor complexes by mediating the formation of a pentameric complex of Ran-GDP, p10, karyopherin-α and -β, and nucleoporin. Dissociation of docked complexes is proposed to be mediated by the association of in situ–generated Ran-GTP with karyopherin-β. According to this model, Ran would function at multiple steps in the translocation process, mediating dissociation of docked complexes along the length of the NPC. Karyopherin-β would have to be regenerated either by GTP hydrolysis (presumably at the cytoplasmic fibers of the NPC) or by exchange of GTP for GDP in the nucleus, mediated by RCC1.

Rates of nuclear import vary depending on physiological conditions (Feldherr and Akin, 1994). According to either model presented above, the relative rates of nuclear import could be limited directly by the concentration of RanGAP1 at the NPC. In addition, the NPC-associated RanGAP1 could also be positioned to control rates of nuclear import indirectly by regulating the concentration of Ran-GTP in the cytosol. Considering the nucleus to be the primary source of Ran-GTP, both the modified 90-kD form of RanGAP1 and Nup358 could be positioned to capture and hydrolyze Ran-GTP diffusing through the NPC. Levels of Ran-GTP in the cytosol are potentially significant, since complexes formed with karyopherin-β could inhibit nuclear import by preventing karyopherin-α/β heterodimers from forming (Rexach and Blobel, 1995). The relative concentrations of RanGAP1 at the NPC could, therefore, be used to regulate the overall rates of nuclear import both directly and indirectly. Conjugation of GMP1 to RanGAP1 correlates with its association with the NPC, and it could in turn serve to regulate the relative concentration of RanGAP1 associated with the NPC.

At least three possible scenarios can be envisioned for how GMP1 may mediate association of RanGAP1 with the NPC. First, GMP1 itself may bind directly to a component of the fibers, thereby tethering RanGAP1 to the NPC. This scenario is not immediately favored, given the detection of GMP1 both in the cytoplasm and in the nucleus, possibly ligated to additional protein substrates. Alternatively, ligation of GMP1 may function to expose a binding site on RanGAP1 that would be masked in the unmodified protein. The 70-kD unmodified form of RanGAP1 forms a homodimer (Bischoff et al., 1994), and GMP1 ligation could potentially disrupt dimer formation, allowing RanGAP1 to interact with a protein at the NPC. It is interesting to note that both RanGAP1 and Nup358 have leucine-rich motifs, which are potential protein–protein interaction domains. Finally, a combination of these two scenarios may occur, whereby ligation of GMP1 creates a binding site with residues from RanGAP1 and GMP1 contributing to association with the NPC. We are currently investigating the details of how modifications of RanGAP1, including phosphorylation and ligation of GMP1, affect the subcellular localization of RanGAP1. In addition to affecting its localization, it is also possible that ligation of GMP1 to RanGAP1 alters its enzymatic activity as a Ran-GTPase-activating protein. Biochemical analysis of the 70-kD unmodified form of RanGAP1 demonstrated it to be a potent RanGTPase activator (Bischoff et al., 1994). At the present time, we have detected significant RanGAP activity associated with isolated nuclear envelopes (Coutavas, E., and G. Blobel, unpublished data), suggesting that the 90-kD modified form of RanGAP1 is also active.

In addition to localization at the NPC, it was also observed that antibodies specific for both RanGAP1 and for GMP1 recognized the mitotic spindle apparatus in dividing cells, suggesting that the 90-kD form of RanGAP1 relocalizes from the NPC to the spindles during mitosis. This finding is particularly interesting in light of the observations that implicate Ran as a regulator of nuclear structure and entry into mitosis (Kornbluth et al., 1994; Clarke et al., 1995). Localization of RanGAP1 at the spindle implicates this structure as a point for the release of Ran-GTP from a mitotic effector molecule. Microtubules may be one such effector of the Ran pathway during mitosis, since overexpression of RCC1 in yeast suppresses mutations of α-tubulin that arrest with excess microtubules (Kirkpatrick and Solomon, 1994). Regulatory interactions (direct or indirect) between Ran and microtubules could, therefore, act as one signal for cell cycle progression. A second potential target of the Ran pathway at the mitotic spindles is NuMA, a 236-kD intranuclear protein that associates with the pericentrosomal domain of the spindle apparatus during mitosis. Ectopic expression of NuMA lacking its globular head domain results in cells with a phenotype morphologically identical to that of temperature-sensitive mutants of

RCC1, and overexpression of wild-type NuMA can partially suppress the phenotype of temperature-sensitive mutant RCC1 cells (Compton and Cleveland, 1993). It remains to be determined whether these properties result strictly from a failure to reimport NuMA into the nucleus after mitosis. Finally, the yeast homologue of GMP1, Smt3p, was originally identified as a suppressor of the *MIF2* gene, whose protein product is a centromere protein required for mitotic spindle integrity (Meluh and Koshland, 1995). It is presently unkown whether Smt3p also localizes to the spindle in yeast and whether it is conjugated to the RanGAP1 homologue. At the present time, immunoblot analysis has revealed only one (apparently unmodified) form of RanGAP1 in *S. cerevisiae* (Hopper et al., 1990; Koepp et al., 1996). If the 90-kD modified form of RanGAP1 proves specifically to localize to the spindles, conjugation with GMP1 may be an important determinant. It is interesting to note that a second yeast ubiquitin-like protein, Dsk2p, has also been identified as a factor involved in mitotic spindle formation (Biggins et al., 1996).

In conclusion, our findings have specific implications for the function of Ran in mediating nuclear import, and for the use of ubiquitin-like modifications to regulate cell processes. We have characterized just one of a new family of ubiquitin-like proteins and found it to be conjugated with relative specificity to RanGAP1. In addition to GMP1, we identified mammalian cDNAs that encode two additional GMP1-related proteins. Given the indications that GMP1 ligation has specific function(s) distinct from the signaling protein degradation of RanGAP1, characterization of these homologues and their substrates will be of interest. At the present time, the enzymology of GMP1 ligation to RanGAP1 is not known, although we have identified an activity that appears to reverse the modification. Surprisingly, this activity was detected in a highly enriched pore complex lamina fraction, indicating that the activity may be associated with the NPC. Because Empigen extraction leads to the solubilization of the majority of proteins associated with the PCLF (with the exception of the lamins and several lamin-associated proteins), it is likely that the putative peptidase is solubilized along with RanGAP1. Solubilization of both substrate and enzyme could allow their interaction and may explain the observed peptidase "activation." While the significance of this peptidase activity is presently unknown, it may potentially control regulated release of RanGAP1 from the NPC. This activity and other regulators of this novel RanGAP1 modification are being investigated.

We thank Erica Johnson for advice and suggestions on experiments and for critical reading of the manuscript. We also thank Helen Shio for expert technical support in preparing and analyzing EM samples, and members of the Rockefeller University Biopolymer Facility, especially Joseph Fernandez, for protein sequence analysis.

This work was supported by the Howard Hughes Medical Institute (G. Blobel and E. Coutavas). M.J. Matunis is an American Cancer Society-Amgen fellow (PF-4195).

Received for publication 5 September 1996 and in revised form 9 October 1996.

Note added in proof. While this manuscript was being reviewed, another group has published the finding that GMP1 interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukemia (Boddy, M.N., K. Howe, L.D. Etkin, E.Solomon, and P.S. Freemont. 1996. *Oncongene.* 13:971–982). Consistent with our immunodetection of GMP1 in brightly stained spots in the nucleus, Boddy et al. demonstrated that GMP1 localizes to distinct structures in the nucleus known as PML nuclear bodies. At the present time, there is no evidence that RanGAP1 also localizes to these structures.

References

Adam, E.J., and S.A. Adam. 1994. Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.* 125:547–555.

Adam, S.A., R.S. Marr, and L. Gerace. 1990. Nuclear protein import in permeabilized mammalian cells requires soluble cytoplasmic factors. *J. Cell Biol.* 111:807–816.

Beddow, A.L., S.A. Richards, N.R. Orem, and I.G. Macara. 1995. The Ran/TC4 GTPase-binding domain: identification by expression cloning and characterization of a conserved sequence motif. *Proc. Natl. Acad. Sci. USA.* 92:3328–3332.

Biggins, S., I. Ivanovska, and M.D. Rose. 1996. Yeast ubiquitin-like genes are involved in duplication of the microtubule organizing center. *J. Cell Biol.* 133:1331–1346.

Bischoff, F.R., C. Klebe, J. Kretschmer, A. Wittinghofer, and H. Ponstingl. 1994. RanGAP1 induces GTPase activity of nuclear Ras-related Ran. *Proc. Natl. Acad. Sci. USA.* 91:2587–2591.

Bischoff, F.R., H. Krebber, T. Kempf, I. Hermes, and H. Ponstingl. 1995*a*. Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. *Proc. Natl. Acad. Sci. USA.* 92:1749–1753.

Bischoff, F.R., H. Krebber, E. Smirnova, W. Dong, and H. Ponstingl. 1995*b*. Co-activation of RanGTPase and inhibition of GTP dissociation by Ran-GTP binding protein RanBP1. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:705–715.

Bischoff, F.R., G. Maier, G. Tilz, and H. Ponstingl. 1990. A 47-kDa human nuclear protein recognized by antikinetochore autoimmune sera is homologous with the protein encoded by RCC1, a gene implicated in onset of chromosome condensation. *Proc. Natl. Acad. Sci. USA.* 87:8617–8621.

Bischoff, F.R., and H. Ponstingl. 1991. Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. *Nature (Lond.).* 354:80–82.

Blobel, G., and V.R. Potter. 1966. Nuclei from rat liver: isolation method that combines purity with high yield. *Science (Wash. DC).* 154:1662–1665.

Bourne, H.R., D.A. Sanders, and F. McCormick. 1991. The GTPase superfamily: conserved structure and molecular mechanism. *Nature (Lond.).* 349: 117–127.

Chen, Z.J., L. Parent, and T. Maniatis. 1996. Site-specific phosphorylation of IκBα by a novel ubiquitination-dependent protein kinase activity. *Cell.* 84: 853–862.

Chi, N.C., E. Adam, and S.A. Adam. 1995. Sequence and characterization of cytoplasmic nuclear protein import factor p97. *J. Cell Biol.* 130:265–274.

Choi, Y.D., and G. Dreyfuss. 1984. Monoclonal antibody characterization of the C proteins of heterogeneous nuclear ribonucleoprotein complexes in vertebrate cells. *J. Cell Biol.* 99:1997–2004.

Ciechanover, A. 1994. The ubiquitin-proteasome proteolytic pathway. *Cell.* 79: 13–21.

Clarke, P.R., C. Klebe, A. Wittinghofer, and E. Karsenti. 1995. Regulation of Cdc2/cyclin B activation by Ran, a Ras-related GTPase. *J. Cell Sci.* 108: 1217–1225.

Compton, D.A., and D.W. Cleveland. 1993. NuMA is required for the proper completion of mitosis. *J. Cell Biol.* 120:947–957.

Coutavas, E., M. Ren, J.D. Oppenheim, P. D'Eustachio, and M.G. Rush. 1993. Characterization of proteins that interact with the cell-cycle regulatory protein Ran/TC4. *Nature (Lond.).* 366:585–587.

Dasso, M. 1993. RCC1 in the cell cycle: the regulator of chromatin condensation takes on new roles. *Trends. Biochem. Sci.* 18:96–101.

Davis, L., and G. Blobel. 1986. Identification and characterization of a nuclear pore complex protein. *Cell.* 45:699–709.

DeGregori, J., A. Russ, H. von Melchner, H. Rayburn, P. Priyaranjan, N.A. Jenkins, N.G. Copeland, and H.E. Ruley. 1994. A murine homolog of the yeast RNA1 gene is required for postimplantation development. *Genes Dev.* 8:265–276.

Dreyfuss, G., S.A. Adam, and Y.D. Choi. 1984. Physical change in cytoplasmic messenger ribonucleoproteins in cells treated with inhibitors of mRNA transcription. *Mol. Cell. Biol.* 4:415–423.

Dwyer, N., and G. Blobel. 1976. A modified procedure for the isolation of a pore complex-lamina fraction from rat liver nuclei. *J. Cell Biol.* 70:581–591.

Egner, R., and K. Kuchler. 1996. The yeast multidrug transporter Pdr5 of the plasma membrane is ubiquitinated prior to endocytosis and degradation in the vacuole. *FEBS Lett.* 378:177–181.

Enenkel, C., G. Blobel, and M. Rexach. 1995. Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. *J. Biol. Chem.* 270:16499–16502.

Feldherr, C.M., and D. Akin. 1994. Role of nuclear trafficking in regulating cellular activity. *Int. Rev. Cytol.* 151:183–228.

Fernandez, J., M. DeMott, D. Atherton, and S. Mische. 1992. Internal protein

FILE

CUTTING EDGE

Protection Against Fas/APO-1- and Tumor Necrosis Factor-Mediated Cell Death by a Novel Protein, Sentrin[1]

Takafumi Okura, Limin Gong, Tetsu Kamitani, Torazo Wada, Izumi Okura, Chik-Fong Wei, Hui-Ming Chang, and Edward T. H. Yeh[2]

Fas/APO-1 and TNF receptor 1 share a common signaling motif in their cytoplasmic tail called the "death domain." Using the death domain as bait in the yeast two-hybrid system, several death domain-containing proteins that participate in cell death signaling have been identified. Here we report the isolation of a novel protein, sentrin, which interacts with Fas/APO-1 and TNF receptor 1 but not with FADD/MORT1 or CD40. Two-hybrid interaction assays reveal that sentrin associates only with the signal-competent forms of Fas/APO-1 or TNF receptor 1 death domains. Sentrin is a novel protein of 101 amino acids with homology to ubiquitin, Nedd8, and a *Saccharomyces cerevisiae* protein, Smt3. When overexpressed, sentrin provides protection against both anti-Fas/APO-1 and TNF-induced cell death. *The Journal of Immunology,* **1996, 157: 4277–4281.**

Nov. 15, No. 10

Fas/APO-1 (CD95) belongs to the TNF receptor superfamily, which is characterized by cysteine-rich pseudorepeats in the extracellular domain (1). Despite the similarity in the organization of the extracellular domain, the cytoplasmic domain of the TNF receptor superfamily is not conserved, implying that different signaling mechanisms must be operative for different receptors. Nonetheless, Fas/APO-1 and TNFR1[3] share a common cytoplasmic signaling motif called the "death domain" (2–4). Deletion or mutation in the death domain abolishes the ability of these receptors to transduce the apoptosis signal. Since the death domain does not contain any obvious kinase or phosphatase motif, its signaling function must be dependent on other associated proteins.

Using the death domains as baits in the yeast two-hybrid system, several laboratories have reported the cloning of novel death domain-associated proteins, including FADD/MORT1, TRADD, and RIP (5–8). After ligation of Fas on the cell surface by either Ab or ligand, a complex called DISC (death-inducing signal complex), which includes Fas, FADD/MORT1, and FLICE/MACH, is formed via death domain or death-effector domain-mediated interaction (9–11). Ligand-induced association of TNFR1 with TRADD, RIP, and FADD/MORT1 has also been demonstrated (7, 12, 13). Taken together, death domain/death domain interaction provide the initial platform for the assembly of signaling complexes that are essential for apoptosis induction or NF-κB signaling. We report the identification of a novel protein, sentrin, which binds to the death domains of Fas/APO-1 and TNFR1 but not that of FADD/MORT1. When overexpressed, sentrin protects cells from both anti-Fas/APO-1 and TNF-induced cell death.

Materials and Methods

Two-hybrid screen and two-hybrid β-galactosidase assay

Yeast strains and shuttle vectors pGBT9 (GAL4 DNA-binding domain) and pGAD424 (GAL4 activation domain) were purchased from Clontech, Palo Alto, CA. The pGBT9-Fas (191–319 aa) was transformed into HF7c using the lithium acetate method. HF7c cells were incubated in 300 ml of YPD medium at 30°C until OD_{600} = 1. The cells were centrifuged at 1000 × *g* for 5 min at room temperature. The harvested cells were suspended in 1.5 ml of 10 mM Tris-HCl-1 mM EDTA-0.1 M lithium acetate solution. pGBT9-Fas (191–319 aa) (0.1 μg) was added into cell suspension together with 100 μg of salmon sperm DNA and 0.6 ml of 10 mM Tris-HCl-1 mM EDTA-0.1 M lithium acetate-40% polyethylene glycol 4000 solution. After incubation at 30°C for 30 min, 700 μl of DMSO were added in the solution. After heat shock at 42°C for 15 min, the cells were harvested and resuspended in 0.5 ml of 10 mM Tris-HCl-1 mM EDTA buffer. The transformed cells were plated on Trp^- synthetic medium and incubated for 4 days at 30°C. The transformed HF7c with pGBT9-Fas IC was cultured in Trp^- synthetic medium and sequentially transformed with 500 μg of the placenta cDNA (Matchmaker; Clontech) which fused to GAL4 DNA-activating domain vector, pGAD10. The co-transformed cells were incubated for 5 days at 30°C on Leu^-, Trp^-, and His^- synthetic medium plates. The positive colonies were picked and restreaked on triple negative plates and assessed for β-galactosidase activity by filter assay as described by the manufacturer. Interaction assays were performed according to the protocols suggested by the manufacturer. Briefly, the yeast transformants were transferred to the paper filters, permeabilized in liquid nitrogen, and then placed on another filter paper presoaked in Z buffer solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 10 mM $MgSO_4$, 50 mM β-mercaptoethanol, and 0.33 mg/ml X-gal). The filters were incubated for up to 24 h at 30°C. The strength of interaction was graded based on the time required for the colony to turn blue. In our hands, the interaction between SV40 T Ag and p53 usually turned blue within 30 min and was graded as +++. The interaction between the death domain of Fas and sentrin (or

Division of Molecular Medicine, Department of Internal Medicine, and Cardiovascular Research Center, Institute of Molecular Medicine for the Prevention of Human Diseases, The University of Texas-Houston Health Science Center, Houston, TX 77030

Received for publication August 8, 1996. Accepted for publication September 23, 1996.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was supported in part by National Institute of Health Grant HL-45851 and an Established Investigator Award from the American Heart Association (to E.T.H.Y.).

[2] Address correspondence and reprint requests to Dr. Edward T. H. Yeh, Division of Molecular Medicine, The University of Texas-Houston Health Science Center, 6431 Fannin, Suite 4200, Houston, TX 77030. Fax 713-792-2373; E-mail address: eyeh@heart.med.uth.tmc.edu.

[3] Abbreviations used in this paper: TNFR1, tumor necrosis factor receptor 1; GST, glutathione *S*-transferase; MBP, maltose-binding protein; aa, amino acids.

Copyright © 1996 by The American Association of Immunologists

0022-1767/96/$02.00

FADD/MORT1) turned blue within 3 h and was graded as ++. The interaction that turned blue between 3–6 h was graded as +. The interaction that required >6 h to turn blue was graded as ±.

Deletion mutants or full length clones were produced by PCR with appropriate primer sets, restriction digested, and ligated to the appropriate vectors. Point mutant was made by oligonucleotide-directed mutagenesis. All constructs were confirmed by DNA sequencing.

In vitro binding

Escherichia coli BL21 cells transformed with pMALc2-sentrin were cultured in LB with 50 μg/ml of ampicillin at OD_{260} = 0.5. After 2 h of incubation from the time of adding isopropyl-β-D-thiogalactopyranoside to a final concentration of 0.1 mM, the culture was harvested, washed in cold buffer (20 mM Tris-HCl, 200 mM NaCl, and 1 mM EDTA), and suspended in 10 ml of buffer to which 1 mM DTT and 0.1 mM PMSF were added. After overnight storage at −20°C, the cells were disrupted by sonication. The lysate was centrifuged for 30 min at 1000 × *g*, and the supernatant was incubated at 4°C for 1 h with 500 μl of a 50% v/v suspension of amylose resin. After centrifugation, the resin was washed four times with 10 ml of buffer. GST-Fas/APO-1, Fas/APO-1(V238N), TNFR1, or TNFR1 Δ20 fusion proteins were produced with the same procedure as with MBP fusion protein except for the use of glutathione-Sepharose beads. After binding, fusion proteins were eluted from glutathione-Sepharose beads in a elusion buffer of 120 mM NaCl, 100 mM Tris-HCl (pH 8.0), and 20 mM reduced glutathione. The eluted fusion proteins were concentrated by Centricon 10 (Amicon). GST-Fas/APO-1, GST-Fas/APO-1 (V238N), GST-TNFR1, and GST-TNFR1 Δ20 (500 ng, each) were incubated with the resin MBP-sentrin (500 ng) in binding buffer (20 mM Tris-HCl (pH 7.4), 100 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM $MgCl_2$, 1 mM DTT, and 0.05% Nonidet P-40) for 12 h at 4°C. After incubation, resins were washed five times in 1 ml of binding buffer. The bound proteins were separated by SDS-PAGE, followed by Western blotting. The blots were probed with goat antiserum against GST (Pharmacia Biotech, Piscataway, NJ) as a primary Ab and with alkaline phosphatase-conjugated rabbit anti-goat Ig as a secondary Ab (The Jackson Laboratory, Bar Harbor, ME). The bound proteins were disclosed by a chemiluminescent detection kit (Tropix Inc., Bedford, MA). Initial experiment revealed that MBP alone count not precipitate the GST fusion proteins under the buffer condition described above.

Cell death protection assay

BJAB (gift of Dr. Fred Wang, Harvard Medical School) and L929 (purchased from the American Type Culture Collection, Rockville, MD) cell lines were cultured in RPMI medium and harvested at log phase. The plasmids for transfection included pSV-β-galactosidase (Promega Corp., Madison, WI), pcDNA3-sentrin, pcDNA3-*crmA*, and pcDNA3 empty vector (Invitrogen, San Diego, CA). pcDNA3-sentrin was constructed by digesting the pGAD424–61ORF with *Bam*HI and subcloned into eukaryotic expression vector pcDNA3 at the *Bam*HI site. Proper orientation of the sentrin insert was confirmed by DNA sequencing. pcDNA3-CrmA was constructed by inserting an *Eco*RI fragment containing *crmA* from a pUC19-*crmA* plasmid construct, a gift of Dr. David Pickup, into the expression vector pcDNA3. BJAB cells (5×10^6) or L929 cells (2×10^6) were transfected with 10 μg of plasmid pSV-β-galactosidase plus equimolar amounts of pcDNA3-sentrin, pcDNA3-*crmA*, or pcDNA3 empty vector by electroporation. For BJAB cells, electroporation was performed at 220 V and 960 μF with a Gene-Pulser I (Bio-Rad, Hercules, CA). After 10 min of incubation at room temperature, cells were resuspended in 1 ml of RPMI medium, transferred to tissue culture flasks containing 20 ml of RPMI medium, and incubated at 37°C. Forty-eight h after transfection, the transfected cells were divided into five equal aliquots, transferred into six-well plates, and treated with medium or anti-Fas/APO-1 (CH11; Panvera, Madison, WI) at 0.25, 2.5, 25, and 250 ng/ml for 20 h. The cells were harvested from the wells, transferred to microfuge tubes, centrifuged, washed with PBS, and lysed in 250 μl of lysis buffer (1 mM DTT-0.2% Triton X-100–0.1 M potassium phosphate, pH 7.8). Ten microliters of the cell lysates were then analyzed in duplicate for β-galactosidase activity using Galacto-Light Plus (Tropix) and a luminescence counter (Packard Instrument Co., Meridien, CT) as described (14). The percentage of survival is calculated by subtracting the chemiluminescence of Ab-treated cells from medium-treated cells divided by the chemiluminescence of medium-treated cells.

For L929 cells, electroporation was performed at 350 V and 500 μF. The transfected cells were divided into four equal aliquots and transferred to a six-well plate. After incubation in regular medium for 48 h, TNF was added to achieve final concentrations of 0.5, 5, and 50 ng/ml. After incubation for another 20 h, the nonadherent cells were gently washed away with PBS, and the adherent cells were harvested from the wells for measurement of β-galactosidase activity as described above. The percentage of survival is calculated by subtracting the chemiluminescence in TNF-treated cells from medium-treated cells divided by the chemiluminescence of medium-treated cells.

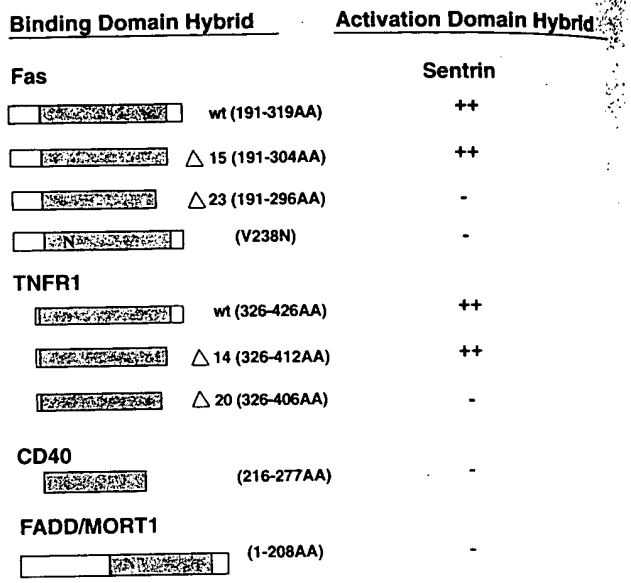


FIGURE 1. Interaction pattern of sentrin. A yeast two-hybrid system was used to map the interactions between sentrin and proteins of interest. Sentrin interacts with the death domains of Fas/APO-1, TNFR1 but not CD40 or FADD/MORT1. The death domain is shaded. ++ indicates positive interaction; − indicates no interaction; N indicates asparagine. Fas/APO-1 and FADD/MORT1 interactions were used as positive controls in all experiments.

Results and Discussion

Using the intracellular domain of Fas/APO-1 (191–319 aa) as a bait in the yeast two-hybrid system, two clones (68 and 61) were isolated from a human placenta cDNA library that interacted strongly and specifically with the bait. DNA sequencing showed that clone 68 encoded an in-frame fusion of the GAL4 activation domain to the death domain of FADD/MORT1 (5, 6). Clone 61, however, encoded an in-frame fusion of the GAL4 activation domain to a novel protein which was named sentrin, after sentry, because it has a guardian function against cell death signaling (see below). Sentrin (1–101 aa) fused to the GAL4 activation domain was used to interact with a panel of Fas/APO-1 mutants fused to the DNA-binding domain. As shown in Figure 1, sentrin interacted with both wild-type and Δ15 mutant of Fas/APO-1, but not with the Δ23 mutant. Furthermore, sentrin could not interact with the human equivalent of the lpr^{cg} mutation, V238N (3). These particular mutants were tested because removal of the C-terminal 15 amino acids of Fas/APO-1 has been shown to enhance cell death signaling, whereas removal of the C-terminal 23 amino acids or substitution of asparagine for valine in position 238 abolished cell death signaling (3). Since sentrin interacted only with the signal-competent forms of Fas/APO-1, this interaction is likely to be functionally relevant.

Because of the homology between the death domains of Fas/APO-1 and TNFR1, we tested whether sentrin could interact with the death domain of TNFR1. As shown in Figure 1, the signal-competent forms of TNFR1, namely wt and Δ14, but not the signal-incompetent form, Δ20, interacted with sentrin (4). Δ14 contains the entire TNFR1 death domain (326–412 aa), which appears to be necessary and sufficient for interaction with sentrin. The strength of interaction between sentrin and Fas/APO-1 vs TNFR1

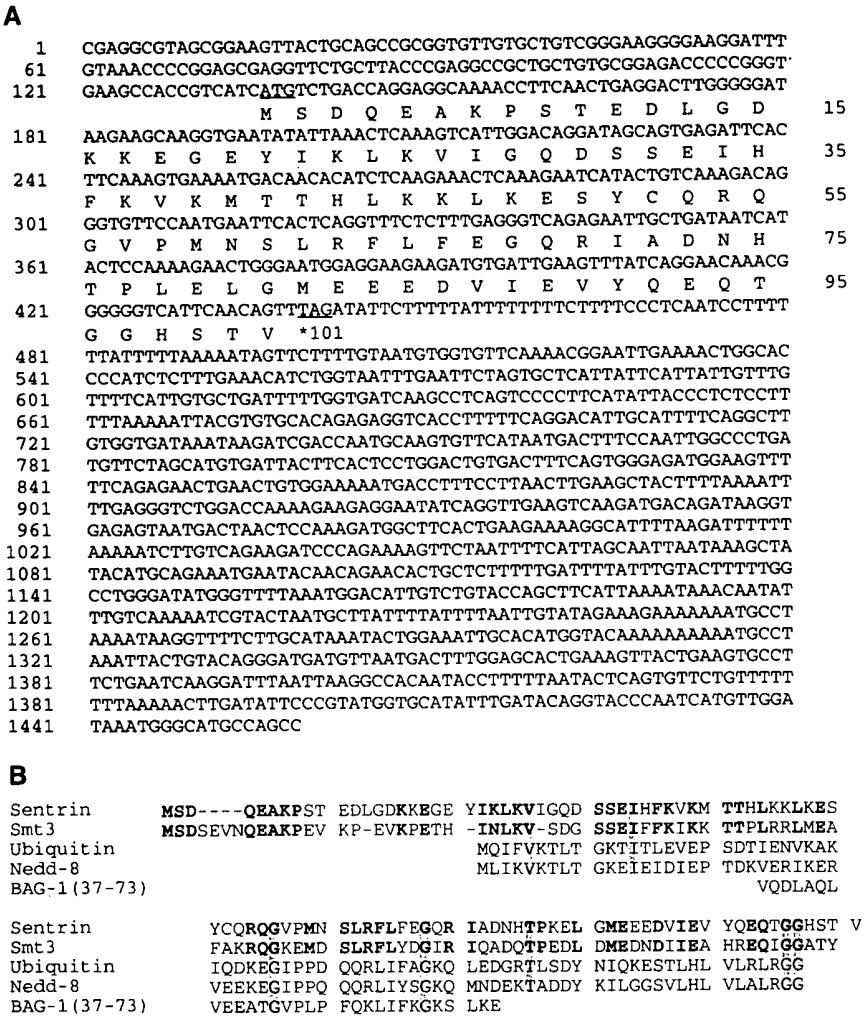


FIGURE 2. *A*, Nucleotide and predicted amino acid sequence of sentrin cDNA. Nucleotides are numbered on the left and amino acid residues are numbered on the right. The start codon ATG and the stop codon TAG are single underlined. Amino acids are indicated in the single letter code. *B*, homology of sentrin to ubiquitin. A BLAST search of the entire data base through the National Center for Biotechnology Information (Bethesda, MD) (31) revealed sequence homology of sentrin with the yeast *S. cerevisiae* Smt3, ubiquitin, Nedd8, the ubiquitin domain to BAG-1 (residues 37–73). Residues identical between sentrin and Smt3 are shown in bold; Residues identical among all sequences are shaded.

appeared to be similar in the yeast two-hybrid system and is comparable to that between Fas/APO-1 and FADD/MORT1. Sentrin, however, could not interact with the death domains of CD40 and FADD/MORT1. Thus, the interaction pattern of sentrin is highly specific.

Northern blot analysis showed that sentrin is expressed in all tissues, but the message level is higher in the heart, skeletal muscle, testis, ovary, and thymus (data not shown). The full length sentrin cDNA contains a 5′-untranslated region of 135 nucleotides and encodes a novel protein of 101 amino acids (Fig. 2*A*). The ATG initiation codon is contained within a Kozak consensus sequence (15), which is necessary for efficient translation. Kyte-Doolittle hydropathy plot analysis showed that sentrin is composed of three hydrophilic domains. The absence of a hydrophobic leader sequence suggests that sentrin is probably a cytosolic protein. BLAST search revealed that sentrin is 18% identical and 48% similar to human ubiquitin (Fig. 2*B*). Sentrin has the same degree of identity and similarity to Nedd8, which was identified by a subtraction cloning approach where Nedd2 (an ICE-like protein) was isolated (16). In addition, sentrin has a weak homology to BAG-1, which contains a short ubiquitin-like domain, binds to Bcl-2, and has an anti-death activity (17). Most interestingly, sentrin is 50% identical and 74% similar to Smt3 from the yeast *Saccharomyces cerevisiae* (P. B. Meluh, unpublished observations; GenBank accession number U27233). Smt3 has the ability to suppress the conditional lethal mif2 mutation which, under nonpermissive temperature, shows increased mitotic chromosome instability, sensitivity to anti-microtubule drugs, and formation of aberrant spindles that break in half during anaphase (18, 19). Yeast cells lacking MIF2 arrest in early mitosis of the cell cycle (18). The high degree of homology between sentrin and Smt3 suggests that these two proteins may have conserved function. Further experiments are needed to fully explore this important insight.

To assess whether the interaction between sentrin and Fas/APO-1 or TNFR1 observed in the two-hybrid system was a direct one, an in vitro interaction assay was conducted. MBP-sentrin, a fusion protein containing the MBP and sentrin, was prepared and used to precipitate a panel of GST fusion proteins containing Fas/APO-1, Fas/APO-1 (V238N), TNFR1, or TNFR1 Δ20. GST fusion proteins were incubated with resin-bound MBP-sentrin in binding buffer for 12 h at 4°C. After incubation, the resins was washed extensively and the bound proteins were separated by electrophoresis on SDS-polyacrylamide gels and transferred to a polyvinylidine difluoride membrane. The blots were probed with goat anti-GST antiserum followed by alkaline phosphatase-conjugated rabbit anti-goat Ig. As shown in Figure 3, sentrin interacted with GST-Fas/APO-1 but not GST-Fas/APO-1 (V238N) and with GST-TNFR1 but not GST-TNFR1 Δ20. Thus, the in vitro interaction results are consistent with those observed in the yeast two-hybrid system.

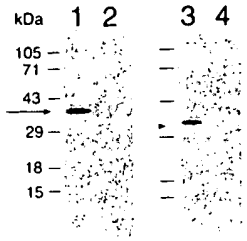


FIGURE 3. In vitro interaction between sentrin and the cell death domains of Fas/APO-1 and TNFR1. *Lane 1,* GST-Fas/APO-1 (175–319 aa); *lane 2,* GST-Fas/APO-1 (V238N); *lane 3,* GST-TNFR1 (326–426 aa), *lane 4,* GST-TNFR1 Δ20 (326–406 aa). The positions of molecular mass markers (in kilodaltons) are shown on the left of the filters. The position of GST-Fas/APO-1 is indicated by an arrow and that of GST-TNFR1 by an arrowhead.

After establishing the association of sentrin and the death domains of Fas/APO-1 and TNFR1, we proceeded to study the functional significance of this interaction in a cell death protection assay. This assay used a co-transfected plasmid with β-galactosidase as a reporter gene. BJAB, a B lymphoma cell line that expresses a high level of Fas/APO-1 and readily undergoes apoptosis after overexpression of FADD/MORT1 or addition of anti-Fas/APO-1 Ab, was used in this transient cell death assay (6, 20). CrmA, a viral serpin inhibitor that has been shown to block apoptosis mediated by anti-Fas/APO-1 Ab, by FADD/MORT1 overexpression, or by TNF treatment (20, 21), was used as a positive control. Empty vector was used as a negative control. BJAB cells were transiently transfected with the empty vector, with a sentrin expression construct, or with a CrmA expression construct in the presence of an equimolar amount of the pSV-β-galactosidase reporter plasmid. Forty-eight hours after transfection, BJAB cells were treated with anti-Fas/APO-1, and cell survival was assessed 20 h later. Viable transfected cells were determined by a sensitive chemiluminescent assay (14). Consistent with previous reports in stably transfected systems (20, 21), transient transfection with the CrmA expression vector significantly suppressed anti-Fas/APO-1-induced apoptosis (Fig. 4*A*). Sentrin expression provided a similar degree of protection against anti-Fas/APO-1-induced cell death compared with CrmA (n = 6).

The protective effect of sentrin against TNF-induced cell death was also investigated. L929, a murine cell line highly sensitive to TNF, was used in this protection assay. Forty-eight hours after electroporation with different plasmids, L929 cells were treated with different concentrations of murine TNF. Eighteen hours later, the nonadherent cells were washed off and the adherent cells were isolated for determination of β-galactosidase activity. As shown, CrmA is protective against TNF-induced cell death (Fig. 4*B*). Sentrin is also protective compared with vector alone (n = 5). The protective effect of human sentrin is less efficient in L929, a murine cell line, compared with BJAB, a human B cell line. Taken together, sentrin protects against both anti-Fas/APO-1 and TNF-mediated cell death.

The mechanism of cell death signaling originated from Fas/APO-1 or TNFR1 is complex (9, 12, 22). It is clear, however, that the death domains of Fas/APO-1 and TNFR1 provide important platforms for protein-protein interaction to occur. The death domain could initiate the recruitment of downstream signaling proteins, such as FADD/MORT1, TRADD, and FLICE/MACH (9–12, 22). The death domain could also recruit kinases or phosphatases that will further modify death domain-associated signaling proteins (8, 23, 24). Thus, cell death domain-associated proteins could have either proapoptotic or antiapoptotic property depending on the intrinsic property of the protein.

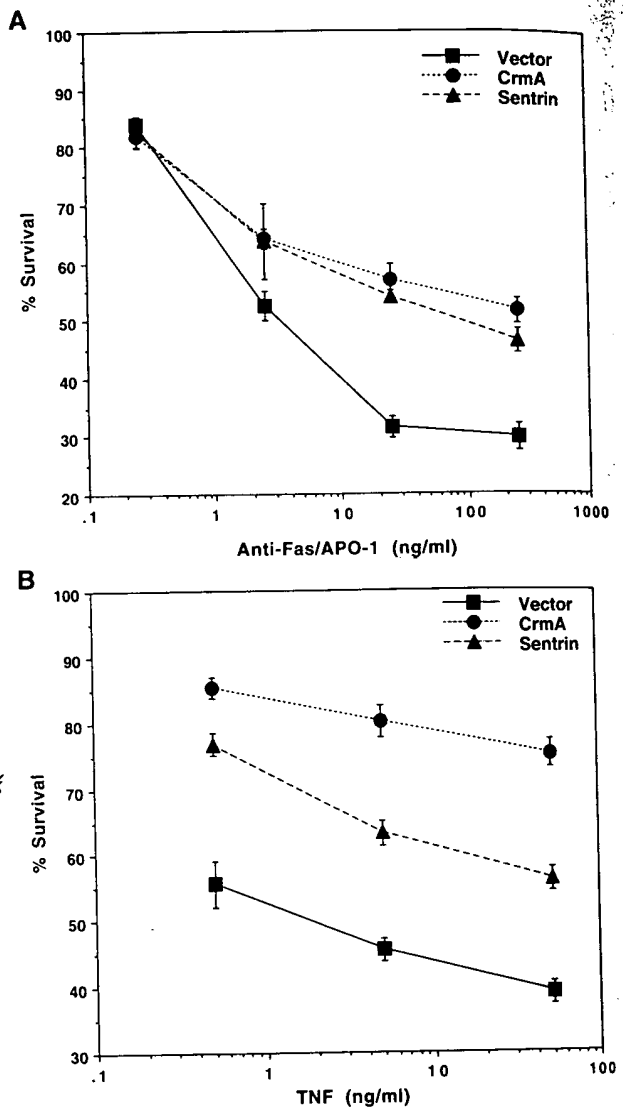


FIGURE 4. Effect of sentrin expression on cell survival after treatments with anti-Fas/APO-1 or TNF. *A,* Anti-Fas/APO-1-mediated cell death in BJAB cells (n = 6); *B,* TNF-induced cell death in L929 cells (n = 5). n = number of independent experiments. Results were expressed as means ± SE.

Several non-death domain-containing proteins that bind to either Fas/APO-1 or TNFR1 have also been reported. FAP-1 is a tyrosine phosphatase that binds to the C-terminal 15 amino acids of Fas/APO-1, a negative regulator of cell death signaling (25). Overexpression of FAP-1 could inhibit Fas/APO-1 signaling. FAF1, another novel protein that binds to the Fas/APO-1 cell death domain, facilitates Fas/APO-1-mediated apoptosis (26). Furthermore, there are a large number of proteins that regulate apoptosis either positively or negatively but do not bind to the cytoplasmic domain of either Fas/APO-1 or TNFR1. They include the IAPs (27), ALG (28), members of the Bcl-2 family (29), and inhibitors of the ICE family, such as CrmA and P35 (30). Full integration of these proteins in the cell death-signaling pathway is yet to be achieved.

Sentrin has several unique features which distinguishes it from other death domain-associated proteins. First, it is a non-death domain-containing protein that binds to both Fas/APO-1 and TNFR1.

FEBS Letters 412 (1997) 102–106

FEBS 18906

Interaction of Fas(Apo-1/CD95) with proteins implicated in the ubiquitination pathway

Karin Becker[1,a], Pascal Schneider[a], Kay Hofmann[b], Chantal Mattmann[a], Jürg Tschopp[a,*]

[a]*Institute of Biochemistry, University of Lausanne, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland*
[b]*Swiss Institute for Experimental Cancer Research, BIL Research Center, Chemin des Boveresses 155, CH-1066 Epalinges, Switzerland*

Received 6 June 1997

Abstract **Fas(Apo-1/CD95), a receptor belonging to the tumor necrosis factor receptor family, induces apoptosis when triggered by Fas ligand. Upon its activation, the cytoplasmic domain of Fas binds several proteins which transmit the death signal. We used the yeast two-hybrid screen to isolate Fas-associated proteins. Here we report that the ubiquitin-conjugating enzyme UBC9 binds to Fas at the interface between the death domain and the membrane-proximal region of Fas. This interaction is also seen in vivo. UBC9 transiently expressed in HeLa cells bound to the co-expressed cytoplasmic segment of Fas. FAF1, a Fas-associated protein that potentiates apoptosis (Chu et al. (1996) Proc. Natl. Acad. Sci. USA 92, 11894–11898), was found to contain sequences similar to ubiquitin. These results suggest that proteins related to the ubiquitination pathway may modulate the Fas signaling pathway.**

© 1997 Federation of European Biochemical Societies.

Key words: Apoptosis; Fas(Apo-1/CD95); Yeast two-hybrid; Ubiquitin

1. Introduction

Apoptosis, or programmed cell death, is essential for the homeostasis of multicellular organisms [1]. Apoptosis is most efficiently induced by Fas (Apo-1/CD95) [2,3]. Fas is a transmembrane receptor of the TNF family of receptor proteins which are characterized by a conserved cysteine-rich extracellular domain [4]. Upon binding of the natural ligand (FasL) or crosslinking with antibodies, cells expressing Fas undergo rapid apoptosis [5,6].

Despite its importance, the mechanisms underlying Fas-mediated apoptosis still remain unclear. Members of the growing family of cysteine proteases specific for aspartic acid (Caspases) appear to be implicated in the execution phase of cell death [7]. Signaling of apoptosis immediately following activation of Fas leads to activation of caspases via the Fas-interacting protein FADD/MORT1 [8,9] and subsequent activation of FLICE/MACH proteases [8,10].

In order to define additional candidate proteins implicated in Fas signaling, we performed a two-hybrid screen using the intracellular domain of Fas as bait. We screened a mouse embryo library and obtained several clones coding for two distinct proteins. One of these represents UBC9, a member of the E2 family of ubiquitin conjugating enzymes.

Ubiquitination of proteins plays a role as a signal for the intracellular degradation of proteins by proteasomes [11,12]. Briefly, in the ubiquitination reaction, ubiquitin is first activated by an activation enzyme, E1. It is then transferred to a cysteine residue of a ubiquitin-conjugating enzyme (E2 enzyme). The E2 enzyme, either alone or together with a ubiquitin-protein ligase, E3, catalyzes the transfer of ubiquitin to a lysine residue of the target protein which is thereby marked for degradation by proteasomes. In yeast, UBC9 represents one out of at least ten different E2 enzymes. It mediates destruction of S- and M-phase cyclins and is essential for viability [13]. The mouse and human UBC9 homologues were recently cloned as proteins interacting with the Rad51 recombination protein [14].

We now show that UBC9 is present in the cytosol and nucleus of transfected cell lines and that it associates with the intracellular domain of the Fas receptor.

2. Material and methods

2.1. Cloning of Fas cytosolic domain fusion proteins

A DNA fragment coding for the cytosolic domain of murine Fas (Ser^{183}–Glu^{306}) was excised *XbaI–PstI* from pKS(+) containing the entire murine Fas [15], and subcloned into PKS(+) (Stratagene). The cytosolic fragment of Fas in this vector from *NotI* (5′) (filled in with Klenow polymerase) to *SalI* (3′) was cloned into the *BamHI* (filled in) and *SalI* sites of the vector PGBT9 (Clontech) thus creating a fusion protein of the Gal4-DNA binding domain and Fas (pGBT-Fas).

A series of fragments and deletion mutations of the Fas cytosolic domain were produced by PCR (using oligonucleotides that contain an added *SmaI* site at the 5′ end or an added *BamHI* site at the 3′ end of the insert) and cloned in frame into the *SmaI* and *BamHI* sites of the Gal4-DNA binding domain vector PGBT9 (Clontech). The following fragments were amplified: Arg^{166}–Glu^{306}, Arg^{166}–Asp^{288}, Leu^{200}–Leu^{289}, Ile^{207}–Leu^{289}, Leu^{289}–Glu^{306}. The Fas cytosolic domain *lpr*^{cg} ($I_{225}N$) (Arg^{166}–Glu^{306}) mutation was created by PCR using mutated oligonucleotides and equally cloned into pGBT9. A further truncated version of Fas was constructed by digestion with *XhoI* (Ala^{220}) and relegation into the *SalI* site of the vector (Arg^{166}–Ala^{220}).

2.2. Two-hybrid screen

A mouse embryo cDNA library cloned into the *NotI*-site of the vector pVP16 [16] which codes for fusion proteins of the viral transcription activation domain VP16 was obtained from S. Hollenberg (Vollum Institute, Portland, Oregon). Yeast strain Y153 [17] was transformed with pGBT-Fas and the library plasmids and analyzed for interaction of the proteins [18]. Mating tests [19] were performed using yeast Y187 (MATα) and Y190 (MATa) transformed with the bait and library plasmids, respectively.

2.3. Transfection of mammalian cells

For expression in mammalian cells, the cDNAs corresponding to mouse UBC9 (R. Bernards, Amsterdam, Netherlands) or fragments thereof were cloned into pCR3 (Invitrogen) and tagged with either a flag or a myc epitope at the N-terminus [20]. The complete cytosolic domain of Fas (Arg^{166}–Glu^{306}) was tagged with a flag epitope at its N-terminus and cloned into pCR3 (Invitrogen). The FADD cDNA in the pcDNA3 vector (Invitrogen) was obtained from J.C. Martinou, Glaxo, Geneva.

*Corresponding author. Fax: (41) (21) 692 5705.
E-mail: jurg.tschopp@ib.unil.ch

[1]*Present address:* MSD Sharp & Dohme, Lindenplatz 1, D-85540 Haar, Germany.

0014-5793/97/$17.00 © 1997 Federation of European Biochemical Societies. All rights reserved.
PII S0014-5793(97)00758-8

Plasmids were transiently transfected into HeLa or 293T cells (293 cells, ATCC CRL 1573, expressing the SV40-T antigen, obtained from M. Peter, Heidelberg) using the calcium-phosphate precipitation method [21]. Protein expression was controlled by separating cell extracts on a polyacrylamide gel and subsequent immunoblotting.

2.4. Immunofluorescence staining

Transfected HeLa cells were grown on sterile coverslips prior to immunofluorescence. Cells were then washed in PBS, fixed in 4% formaldehyde and permeabilized with 0.1% saponin in PBS. Transfected proteins were stained with monoclonal antibody to the flag epitope (M2, Kodak). After extensive washing, an antibody against mouse immunoglobulins conjugated to FITC (Dianova, Hamburg, Germany) was added. Slides were mounted using FluorSave Reagent (Calbiochem) and analyzed using a Zeiss Axiophot microscope.

2.5. Immunoprecipitation

293T cells were transfected with flag-tagged Fas cytosolic domain and myc-tagged UBC9 in a 25 cm^2 plate. Cells were lysed in 1 ml lysis buffer [22] and precipitated with a monoclonal antibody to the flag tag coupled to agarose (M2, Kodak). Co-precipitation of myc-tagged UBC9 was analyzed by Western blot using the monoclonal anti-myc antibody 9E10 (gift from R. Iggo, ISREC, Epalinges). Anti-FADD monoclonal antibodies were purchased from Transduction Laboratories, Lexington.

3. Results

3.1. Identification of UBC9 as a protein binding partner of Fas

We used the yeast two-hybrid system to screen for proteins that interact with the cytosolic domain of the Fas/Apo-1 receptor. As bait we fused the fragment of Fas from Ser^{183} to Glu^{306} to the Gal4 DNA binding domain. This plasmid was transformed into yeast strain Y153 along with a library of mouse embryo cDNA fused to the transcription activation domain VP16 [22]. Twenty-seven positive clones were obtained from 5×10^6 clones screened. Sequencing of the inserts revealed that four inserts corresponded to the mouse homologue of the *S. cerevisiae* ubiquitin-conjugating enzyme UBC9 [13,14]. Three of the clones were identical. All of the clones lacked the 2 C-terminal amino acids of full-length UBC9, and one clone additionally lacked 6 amino acids at the N-terminus. The full-length mouse UBC9 cDNA (obtained from R. Bernards) was subsequently cloned into the vectors for the two-hybrid system to confirm the results. UBC9 interacted specifically with Fas (Fig. 1), but not with the proteins lamin A, LDL receptor, or poly Ig receptor (not shown) used as control.

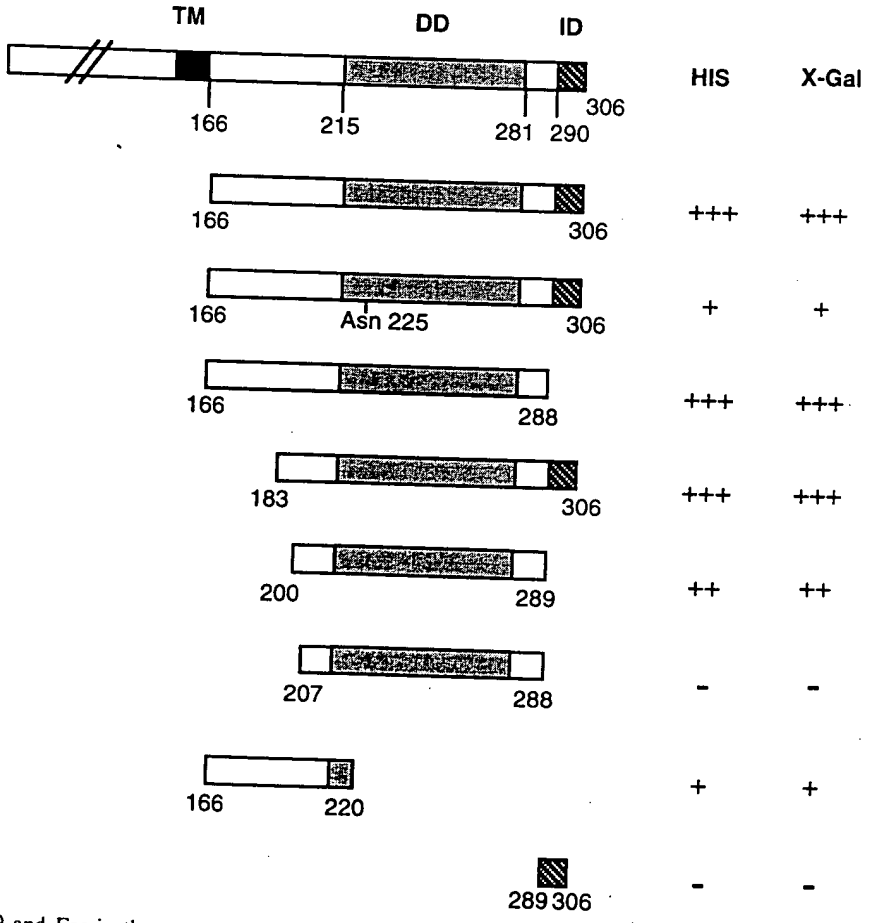


Fig. 1. Interaction of UBC9 and Fas in the yeast two-hybrid system. TM, transmembrane segment of Fas; DD, death domain; ID, inhibitory domain, binds to FAP-1. Several deletion mutations of the cytosolic domain of Fas were cloned as fusion proteins with the DNA binding domain of Gal4. Interaction of these with the VP16 fusion protein of UBC9 was tested in the two-hybrid system. Growth on histidine-lacking media and β-galactosidase activity is indicated. +++: strong reaction (large colonies, dark blue 213color), +: weak interaction (small colonies, faint blue color).

3.2. Localization of the Fas-UBC9 interaction site

In order to characterize the interactions of UBC9 and Fas more precisely, we constructed several truncated versions of the Fas cytosolic domain (Fig. 1). Both the complete UBC9 protein and the fragments obtained in the two-hybrid screen interacted very strongly with the entire cytosolic domain of Fas (Arg^{166}–Glu^{306}) and with the fragments which lacked either 17 amino acids adjacent to the membrane-interacting segment (Ser^{183}–Glu^{306}) or the C-terminal 18 amino acids (Arg^{166}–Asp^{288}) (Fig. 1). Neither of the UBC9 proteins interacted with the fragment of Fas corresponding to the death domain (Ile^{207}–Asp^{288}) alone or with the C-terminal 18 amino acids (Leu^{289}–Glu^{306}) known to interact with FAP-1 [23]. In contrast, weak but detectable interaction was found with fragments in which the death domain was flanked by additional 15 amino acids at its N-terminus (Leu^{200}–Leu^{289}) or when the fragment contained the complete membrane proximal part, but only 7 residues of the death domain (Arg^{166}–Ala^{220}). In addition, UBC9 interacted only weakly with the cytosolic domain of Fas which contained a mutation in the death domain corresponding to the *lpr*^{cg} [24] mutation in mice which renders Fas inactive [25]. From these experiments, we conclude that efficient binding of UBC9 to the cytosolic domain of Fas requires both a functional death domain and part of the membrane proximal segment.

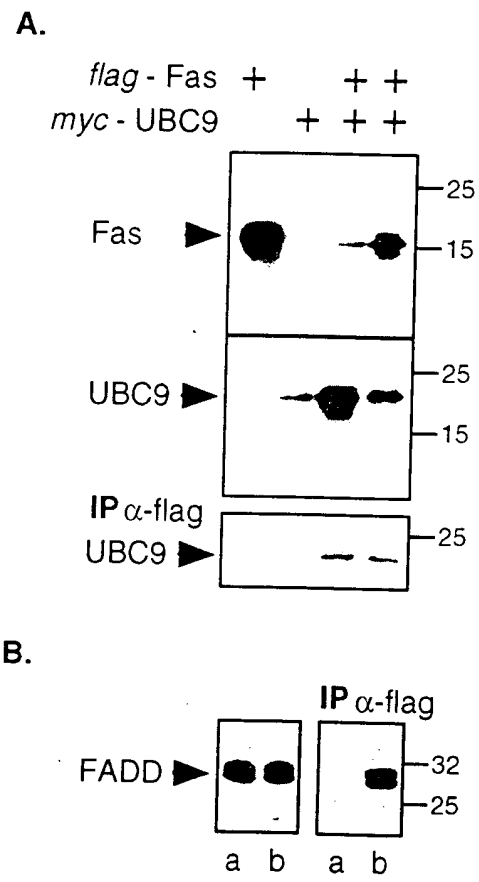


Fig. 2. Interaction of UBC9 and Fas in mammalian cells. (A) 293T cells were transfected with, from left to right, (a) flag-tagged Fas cytosolic domain (10 μg plasmid), (b) myc-tagged UBC9 (2 μg), (c) flag-tagged Fas cytosolic domain (2 μg plasmid) and myc-tagged UBC9 (8 μg), and (d) flag-tagged Fas cytosolic domain (8 μg plasmid) and myc-tagged UBC9 (2 μg). Cells were lysed and postnuclear supernatant analyzed by Western blot (top two panels) using Fas-detecting anti-flag and UBC9-detecting anti-myc antibodies, respectively. In the third panel, experiments are shown where Fas was first precipitated (IP) from lysates of transfected cells with anti-flag antibodies bound to agarose. Precipitates were subsequently analyzed by Western blot for the co-precipitation of myc-tagged UBC9. (B) Control experiment: interaction of FADD and Fas in mammalian cells. 293T cells were either transfected with FADD cDNA alone (a) or co-transfected with FADD and flag-tagged Fas cytosolic domain (b). The left panel shows a Western blot of lysates of transfected cells using an anti-FADD antibody. In the right panel (IP), Fas, when present, was first precipitated (IP) with anti-flag antibodies bound to agarose. Precipitates were subsequently analyzed by Western blot for the co-precipitation of FADD.

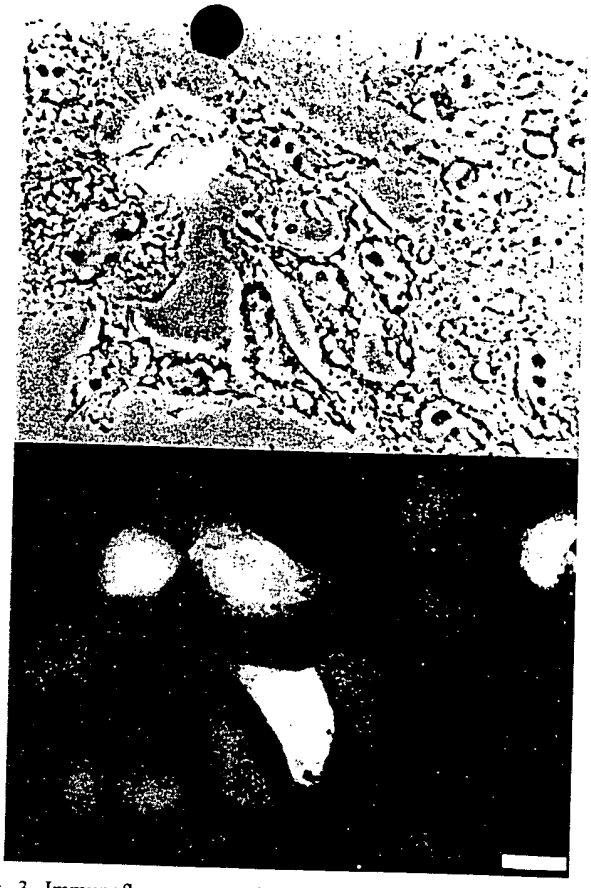


Fig. 3. Immunofluorescence of UBC9. HeLa cells were transfected with UBC9 tagged with a flag epitope at its N-terminus, fixed on a coverslip, and stained with anti-flag antibody (lower panel). The corresponding phase-contrast image is shown in the upper panel. The bar corresponds to a distance of 15 μm.

3.3. Interaction of UBC9 and Fas in vivo

To confirm that interaction of Fas with UBC9 occurs in vivo, 293T human embryo kidney cells were co-transfected with a plasmid coding for the cytosolic domain of Fas tagged with a flag epitope and a plasmid coding for mouse UBC9 tagged with a myc epitope. Cells were lysed and subjected to immunoprecipitation using an anti-flag epitope antibody. The immunoprecipitates were analyzed by Western blot using an antibody against the myc epitope. UBC9 was only precipitated if it was coexpressed with Fas (Fig. 2A). If the two proteins were expressed separately and the cell lysates were mixed before immunoprecipitation, no binding was observed (data not shown) indicating that Fas and UBC9 interaction is weak. As expected, the transiently expressed cytoplasmic segment of Fas strongly interacted with FADD/Mort1 (Fig. 2B).

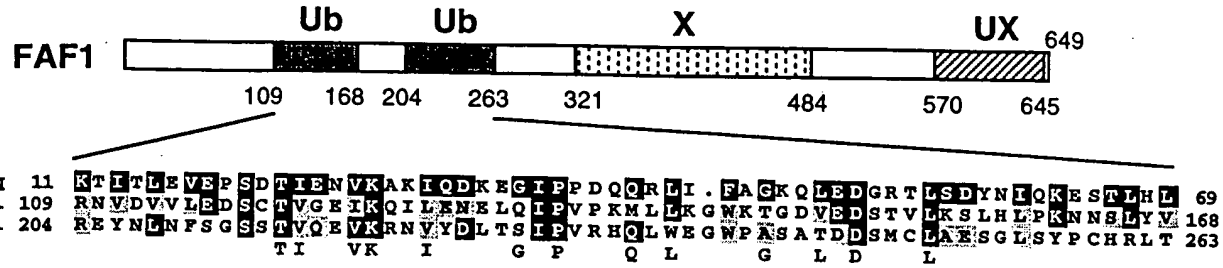


Fig. 4. Sequence homology of FAF1 with proteins involved in the ubiquitin pathway. For establishing the homology, the generalized profile method was used. Alignment of the two regions of FAF1 homologous to ubiquitin (ubiq) is shown. The most conserved amino acids in ubiquitin-like proteins are listed below the alignment. Black-boxed residues correspond to identical amino acids in at least two of the three sequences shown, shaded amino acids represent conserved residues. X = homology with *C. elegans* ORF C281.1; UX = homology with proteins involved in ubiquitination pathway. Accession numbers: mouse FAF1: gb: U39643, human ubiquitin: P02248 (76 aa), C28g1.1: gb: U41026.

3.4. UBC9 is present in the cytosol and the nucleus

Yeast UBC9 has been reported to be active in the nucleus of yeast [13]. Since our experiments indicated that UBC9 was interacting with the cytoplasmic domain of Fas, it was important to investigate whether UBC9 was also present in the cytoplasm of mammalian cells. The flag-tagged UBC9 expression construct was transiently expressed in HeLa cells and localization of the protein was analyzed by immunofluorescence. As shown in Fig. 3, UBC9 was expressed in the cytosol of transfected cells. A considerable portion was also found in the nucleus suggesting that the protein shuttles between the two cellular compartments. The same result was obtained with a myc-tagged version of UBC9 in COS cells (data not shown). This localization is consistent with the binding of UBC9 to the cytosolic domain of Fas.

4. Discussion

Using the two-hybrid screen, we have identified the ubiquitin-conjugating enzyme UBC9 as a Fas-interacting protein, increasing to date the number of proteins known to associate with the cytoplasmic segment of Fas to five. This finding is in agreement with recently published data by Wright et al. [26], who reported that Fas interacts with UBC-FAP (identical to UBC9). In addition to UBC9, two death domain containing proteins, i.e. FADD/MORT1 [8,9] and RIP [27], specifically interact with the death domain of Fas. Whereas the role of FADD/MORT1 is to physically link the FLICE/MACH proteases to Fas and thus initiate apoptosis [10,28], RIP's function in Fas-mediated cell death is less clear as it preferentially associates with TNFR1 via the death domain protein TRADD [29]. Fas also interacts with two proteins that lack death domains, i.e. FAF1 and FAP-1. FAF1, if overexpressed in mammalian cells, potentiates apoptosis [30], in contrast to the phosphatase FAP-1 which has been reported to act as inhibitor of cell death [31]. FAP-1 binds to the carboxyl-terminal 15 amino acids of Fas, a sequence known to represent a negative regulator of Fas-induced apoptosis [32]. The FAF1 binding region in Fas has not been mapped precisely. Similar to UBC9, FAF1 is unable to interact with the Fas *lpr*^{cg} mutation and no homology with any known sequence in the data banks was found. Using a generalized profile method [33], however, sequence similarity with several sequence motifs present in proteins of the ubiquitination pathway became apparent (Fig. 4). First, one repeated motif homologous to ubiquitin itself is present in the N-terminus of FAF1. Second, the C-terminus comprises a UX sequence motif present in proteins implicated in ubiquitin conjugation [34]. Finally, the middle region of FAF1 shows sequence similarity with a motif (X) found in the *C. elegans* ORF C28G1.1 where this (X) motif is found adjacent to a sequence homologous to UBC9.

The physiological relevance of the association of Fas intracellular domain with two proteins apparently implicated in the ubiquitination pathway remains to be elucidated. When FAF1 is overexpressed, the percentage of apoptotic cells increases through Fas signaling, suggesting that it is a positive regulator of apoptosis. We have observed no effect on overexpression of UBC9 in mammalian cells, probably reflecting the high endogenous levels already present in most mammalian systems [14]. There are several possible explanations for the UBC9/Fas interaction. First, UBC9 may be directly involved in ubiquitin-dependent degradation of Fas by proteasomes. Fas signaling leads to rapid apoptosis and one mechanism to control this potentially dangerous death pathway could be to limit the half-life of the receptor. Although nothing is known on the stability of Fas and possible ubiquitination, the related TNFR1 appears to be short-lived [35]. Interestingly, TNFR was shown to be ubiquitinated [36] and to bind a protein related to a proteasome subunit upstream of the death domain [35,37]. Second, Fas may target UBC9 to membrane sites where ubiquitination of neighboring proteins may trigger events required for apoptosis. Proteasome proteolytic activity is required for certain apoptotic pathways in neurons and thymocytes [38,39]. However, modification of proteins by ubiquitin can have consequences other than direct targeting to the 26S proteasome. Ubiquitination may act as a signal for receptor endocytosis to lysosomes as recently reported for a yeast plasma membrane receptor [40]. Moreover, the biosynthesis or activity of several receptor proteins has been shown to be regulated by ubiquitination, as in the case of the cystic fibrosis transmembrane regulator, the T-cell receptor, the platelet-derived growth factor receptor and an IκBα protein kinase [41]. Understanding the role of the ubiquitination pathway related proteins FAF1 and UBC9 in Fas signaling will certainly lead to a better understanding of how apoptotic signals are modulated.

Acknowledgements: K.B. is supported by a HFSO long-term fellowship. The authors thank Kai Matuschewski and Stefan Jentsch (University of Heidelberg, Germany) and Terra Arnason (University of München, Germany) for helpful discussions. We gratefully acknowledge the help of Paul Bartel (University of Washington, Seattle, USA) in providing yeast strain Y153, of Rene Bernards (Netherlands Cancer Institute, Amsterdam, Netherlands) who provided the plasmid encoding mouse UBC9, and of Stanley Hollenberg (Vollum Institute, Port-

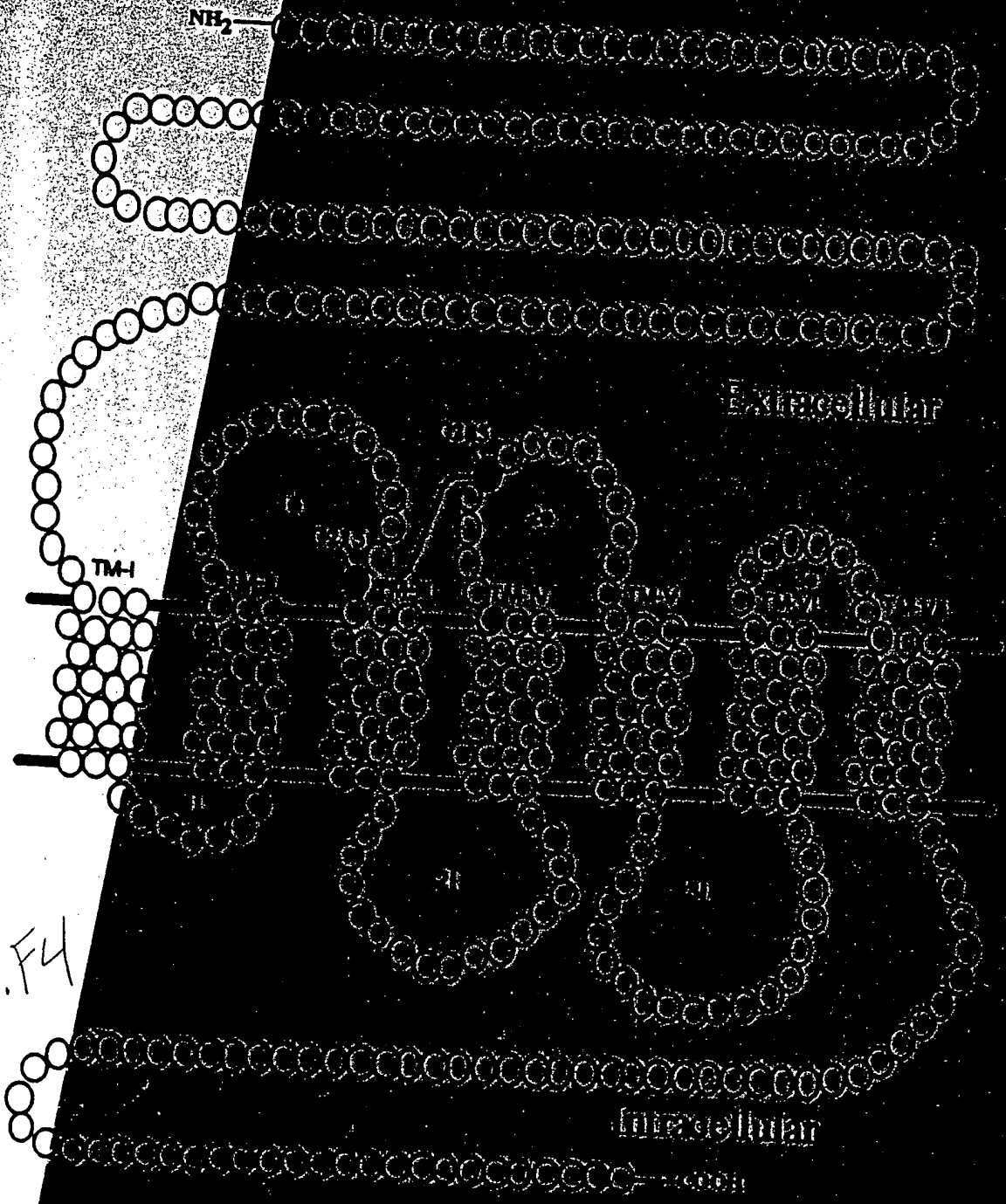
land, OR, USA) who provided the mouse embryo library in pVP16. We also thank Paul Bartel (University of Washington, Seattle, USA), Robin Brown and Stuart Farrow (Glaxo Wellcome, Stevenage, UK) for many helpful discussions while setting up the two-hybrid system. This work was supported by the Swiss National Foundation (to J.T.).

References

[1] Wyllie, A.H., Kerr, J.F. and Currie, A.R. (1980) Rev. Cytol. 68, 251–251.
[2] Itoh, N. et al. (1991) Cell 66, 233–243.
[3] Trauth, B.C., Klas, C., Peters, A.M., Matzku, S., Moller, P., Falk, W., Debatin, K.M. and Krammer, P.H. (1989) Science 245, 301–305.
[4] Smith, C.A., Farrah, T. and Goodwin, R.G. (1994) Cell 76, 959–962.
[5] Dhein, J., Daniel, P.T., Trauth, B.C., Oehm, A., Moller, P. and Krammer, P.H. (1992) J. Immunol. 149, 3166–3173.
[6] Tanaka, M. et al. (1996) Nature Med. 2, 317–322.
[7] Dixit, V.M. (1996) Curr. Biol. 6, 555–562.
[8] Boldin, M.P., Varfolomeev, E.E., Pancer, Z., Mett, I.L., Camonis, J.H. and Wallach, D. (1995) J. Biol. Chem. 270, 7795–7798.
[9] Chinnaiyan, A.M., O'Rourke, K., Tewari, M. and Dixit, V.M. (1995) Cell 81, 505–512.
[10] Muzio, M. et al. (1996) Cell 85, 817–827.
[11] Seufert, W. and Jentsch, S. (1992) EMBO J. 11, 3077–3080.
[12] Ciechanover, A. (1994) Cell 79, 13–21.
[13] Seufert, W., Futcher, B. and Jentsch, S. (1995) Nature 373, 78–81.
[14] Kovalenko, O.V., Plug, A.W., Haaf, T., Gonda, D.K., Ashley, T., Ward, D.C., Radding, C.M. and Golub, E.I. (1996) Proc. Natl. Acad. Sci. USA 93, 2958–2963.
[15] Watanabe-Fukunaga, R., Brannan, C.I., Itoh, N., Yonehara, S., Copeland, N.G., Jenkins, N.A. and Nagata, S. (1992) J. Immunol. 148, 1274–1279.
[16] Vojtek, A.B., Hollenberg, S.M. and Cooper, J.A. (1993) Cell 74, 205–214.
[17] Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H. and Elledge, S.J. (1993) Genes Dev. 7, 555–569.
[18] Bartel, P.L. and Fields, S. (1995) Methods Enzymol. 254, 241–263.
[19] Guthrie, C. and Fink, G.R. (1991) Methods Enzymol. 194, 1–932.
[20] Prickett, K.S., Amberg, D.C. and Hopp, T.P. (1989) Biotechniques 7, 580–589.
[21] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) in: Current Protocols in Molecular Biology, J. Wiley and Sons, New York.
[22] Rothe, M., Pan, M.G., Henzel, W.J., Ayres, T.M. and Goeddel, D.V. (1995) Cell 83, 1243–1252.
[23] Sato, T., Irie, S., Kitada, S. and Reed, J.C. (1995) Science 268, 411–415.
[24] Kimura, M. and Matsuzawa, A. (1994) Int. Rev. Immunol. 11, 193–210.
[25] Nagata, S. and Golstein, P. (1995) Science 267, 1449–1456.
[26] Wright, D.A., Futcher, B., Ghosh, P. and Geha, R.S. (1996) J. Biol. Chem. 271, 31037–31043.
[27] Stanger, B.Z., Leder, P., Lee, T.H., Kim, E. and Seed, B. (1995) Cell 81, 513–523.
[28] Boldin, M.P., Goncharov, T.M., Goltsev, Y.V. and Wallach, D. (1996) Cell 85, 803–815.
[29] Hsu, H.L., Huang, J.N., Shu, H.B., Baichwal, V. and Goeddel, D.V. (1996) Immunity 4, 387–396.
[30] Chu, K., Niu, X. and Williams, L.T. (1995) Proc. Natl. Acad. Sci. USA 92, 11894–11898.
[31] Sato, T., Irie, S., Kitada, S. and Reed, J.C. (1995) Science 268, 411–414.
[32] Itoh, N. and Nagata, S. (1993) J. Biol. Chem. 268, 10932–10937.
[33] Bucher, P. and Bairoch, A. (1994) in: Proceedings Second International Conference on Intelligent Systems for Molecular Biology (Altman, R., Brutlag, D., Karp, P., Lathrop, R. and Searls, D., Eds.) p. 53, AAAI Press, Menlo Park.
[34] Hofmann, K. and Bucher, P. (1996) Trends Biochem. Sci. 21, 172–173.
[35] Boldin, M.P., Mett, I.L. and Wallach, D. (1995) FEBS Lett. 367, 39–44.
[36] Loetscher, H., Schlaeger, E.J., Lahm, H.W., Pan, Y.C., Lesslauer, W. and Brockhaus, M. (1990) J. Biol. Chem. 265, 20131–20138.
[37] Dunbar, J.D., Song, H.Y., Guo, D., Wu, L.W. and Donner, D.B. (1997) J. Immunol. 158, 4252–4259.
[38] Grimm, L.M., Goldberg, A.L., Poirier, G.G., Schwartz, L.M. and Osborne, B. (1996) EMBO J. 15, 3835–3844.
[39] Sadoul, R. et al. (1996) EMBO J. 15, 3845–3852.
[40] Hicke, L. and Riezman, H. (1996) Cell 84, 277–287.
[41] Hochstrasser, M. (1996) Cell 84, 813–815.

FEBS LETTERS

AN INTERNATIONAL JOURNAL FOR THE RAPID PUBLICATION OF SHORT REPORTS IN BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR CELL BIOLOGY



QP501.F4

Published by Elsevier on behalf of the Federation of European Biochemical Societies

Volume 412, Number 1, 21 July 1997
FEBLAL 412 (1) 1–256

ISSN 0 014 5793